SURFACE CHARACTERISTICS OF BRASSICA LEAVES AND THEIR INFLUENCE ON INFECTION BY FUNGAL PATHOGENS

by

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Dedicated to my mother and sister, without whose love and support this thesis would not have been possible

DECLARATION

This is to declare this thesis has been carried out by myself and has not been accepted in any previous application for a degree. All information and assistance obtained from other sources has been acknowledged in the appropriate places.

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ABSTRACT

The role epicuticular waxes play in resistance of brassicas to foliar diseases was investigated for two known brassica pathogens, *Alternaria brassicicola* and *Erysiphe cruciferarum*, a weak parasite *Alternaria alternata* and a non-pathogen *Erysiphe graminis*. Waxes of swede, oilseed rape and Brussels sprout leaves from different leaf positions were found to be composed of eight analogous compounds but in varying proportions. Epicuticular waxes from seven Brussels sprout mutant lines, in comparison to a commercial variety, again had eight similar groups of compounds, however the variations in proportions were more distinct. Correlations could be made between prevalence of certain compound groups and wax crystal configuration. Waxes containing high ketones and hydrocarbons had crystals in the form of rods which projected from the cuticle and these surfaces tended to be waxy. Conditions in which the brassicas were grown could modify crystal morphology. Temperature was seen to transform the crystalline configuration of wax crystals, whereas light intensity altered their dimensions.

Waxy surfaces had the lowest wettability and permeability, and also tended to be most resistant to *Alternaria* infection. Infection by *Erysiphe cruciferarum* seemed to be related to the genetic background of the host and not surface phenotype. Treatment of the surface with surfactant, which reduced the wettability, and environmental conditions which increased wettability had equivalent effects on *Alternaria* infection but variations in *Erysiphe* infections could not be explained by changes in leaf surface wettability.

Microscopic examination of leaf surface behaviour and early penetration

events revealed that the varying features of the different brassica leaf surfaces, or even a non-host surface in the case of *Erysiphe cruciferarum,* did not greatly affect extra-matrical development of the *Alternaria* pathogens or *Erysiphe cruciferarum*. Variations seen in numbers of sub-cuticular hyphae, originating from successful penetrations of the cuticle by *A. brassicicola* and *A. alternata,* correlated with the degree to which the host was infected with blackspot. With *Alternaria*, the penetration phase seemed to be crucial as the determinant of successful infection. The significant phase for successful infection by *Erysiphe cruciferarum* seemed to be the fungal-host interface. Resistance was of a quantitative nature and was expressed by the extent of colony development.

When the growth of *Erysiphe graminis*, was observed on swede leaves, it was found that the infection course of the pathogen was only moderately affected by the leaf surface. Many conidia attempted penetration and several formed rudimentary haustoria within the epidermal cells. It is concluded that the epicuticular waxes confer a degree of resistance to *Alternaria* pathogens, but play a limited role in resistance to powdery mildew.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The contribution of passive defence mechanisms on leaf surfaces to disease control of foliar pathogens has attracted scientific interest over at least the last 100 years. However, due to inadequate technology pioneer researchers did not have the adequate means to study and fully explain these interactions. Examination of plant-pathogen interrelationships since about the middle of the century concentrated on the events taking place after the pathogen had penetrated into plant tissues. Even though deposition and penetration are two critical stages in the development of any pathogen this area was neglected. Developments in electron microscopy and biochemical methods are now leading to an improved understanding of the nature of plant surfaces (see for example Martin & Juniper, 1970; Juniper & Jeffree, 1983). Moreover, studies on several aspects of the interplay of pathogen and host at the leaf surface appear to have increased in more recent years, for example with respect to thigmotropism (Wynn & Staples, 1981) and to the phyllosphere as a theatre of microbial interactions (Dickenson & Preece, 1976; Fokkema & Van Den Heuvel, 1986). However, many areas still remain where knowledge is inadequate. For a comprehensive understanding of plant-pathogen interrelationships, there continues to be a need for further systematic research on the surface layers of plants and their significance in infection processes. It was in recognition of this need that the present studies were initiated.

Apart from posing questions of general biological interest, a consideration of plant surfaces in relation to plant diseases is of potential practical value in the development of disease control strategies, based on the

manipulation of plant resistance mechanisms. Work on disease resistance mechanisms has tended to focus on active defences i.e. "the series of interconnective processes which, following recognition, are induced in the host cell through its continuing irritation by structures or products of the parasite and which results in exclusion, inhibition or elimination of the potential pathogen" (Heitefuss, 1980).

Such forms of resistance, involving hypersensitivity and phytoalexin production, have been exploited widely in practice. However they are generally under oligogenic control and race-specific (Simmonds, 1979) and they are frequently found to be short-lived in their effectiveness in commercial practice. More attention is now being paid to more durable forms of resistance derived from passive defence mechanisms. Passive resistance factors which lie in the surface layers of the plant may present the first line of defence against pathogen attack.

Invasion of a plant by a pathogen is dependent in the first instance on the arrival and retention of inoculum on the plant. Effective inoculum deposition is influenced by the general form and growth habit of the plant as well as by the structural features of its surface (Royle, 1976). The deposition phase, however, is outside the scope of present work which is concerned with the events following deposition up to penetration of the surface and the ensuing interactions with the underlying cells.

The study is focussed on leaf surface characteristics of brassica plants in relation to infection by fungal pathogens. The genus *Brassica* is of great economic importance, containing a large number of vegetable crop plants as well as plants providing animal foodstuffs and oil seeds. There are several reports implicating leaf surface factors in the resistance of brassica plants to

foliar pathogens. According to Maddock, Ingram & Gilligan (1981), cuticular characteristics are responsible for resistance to light leaf spot (*Pyrenopeziza brassicae*) in several brassicas which they examined. Rawlinson, Muthyalu & Turner (1978) related the severity of light leaf spot to the effect of herbicides on the epicuticular wax on oilseed rape. Higher levels of infection by *Alternaria* in brassicas have been associated with the use of surfactants (Munro, 1984) or wiping leaves (Tewari & Skoropad, 1976) both of which alter the surface characteristics. Mhunde & Bhowmik (1985) attributed the low incidence of grey leaf spot (*Alternaria brassicae*) in resistant cultivars of mustard (*Brassica oleracea* var. *alboglabra*) and rape (*Brassica napus*) to cuticle factors. Prasanna (1984) attributed variation in resistance over a wide range of different brassicas to infection by *Alternaria brassica*e and *Alternaria brassicicola* to differences in their surface wax layers, and showed that rubbing the surface to alter or remove this layer increased infection rates of these pathogens.

Three brassica plants were selected for this investigation. Swede (*Brassica napus* L. ssp *rapifera* (Metzg.) Sinsk.) cv. Doon Major, Winter oilseed rape (*Brassica napus* L. ssp *oleifera* (Metzg.) Sinsk.) cv. Jet Neuf and Brussels sprout (*Brassica oleracea* L. var. *gemmifera* DC.) cv. Cambridge N^{o}5. In addition a cultivar of barley (*Hordeum vulgare* L.), Golden Promise, was used in non-host studies.

With respect to fungi *Alternaria brassicicola* (Schwein.) Wiltshire responsible for dark leaf spot (blackspot) and *Erysiphe cruciferarum* Opiz ex Junell causing powdery mildew were chosen as commonly occurring, recognised pathogens of brassicas while *Alternaria alternata* (Fr.) Keissler was also included as a saprophyte or weak parasite associated with a wide range of plants. In addition, *Erysiphe graminis* DC. f.sp. *hordei* was used as a

specialised pathogen of barley, but acting as a non-pathogen to brassicas.

Following a description of general materials and methods the experimental work is reported in three sections, the first dealing with the physical and chemical attributes of brassica leaf surfaces (Chapter 3), the second with assessments of disease development on brassica leaves in relation to their surface characteristics (Chapter 4) and the third with microscopic studies of pathogen development in relation to leaf surface characteristics (Chapter 5).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2. GENERAL MATERIALS AND METHODS

2.1. Growth conditions of plant material.

Plants of swede (*Brassica napus* ssp. *oleifera*) cv. Doon Major, oilseed rape (*Brassica napus* ssp. *rapifera*) cv. Jet Neuf and Brussels sprout (*Brassica oleracea* var. *gemmifera*) cv. Cambridge N°·5 were grown from seed in 7 cm pots in a peat based compost (Appendix 2.1). Plants were raised in a heated glasshouse maintained at 24 ± 4 °C. Supplementery light (14 h daily) was provided by high pressure mercury lamps suspended 1.5 m above the plants. After 3-4 weeks seedlings were transplanted to 15 cm pots and allowed to grow for a further 4-5 weeks when they were sampled.

2.2. Growth conditions of plant material in environment cabinets.

Swede, oilseed rape and Brussels sprout plants were sown as before but were raised in Di Roma Cryoking growth rooms, set at 18 and 12 °C respectively. Within each growth room movable shelf units were constructed at split levels. Pots were placed on each shelf such that radiant energies of 9000 lux were measured at the upper shelves and 4500 lux at the lower shelves (measured at plant apices). Light was provided by Hermes 3 Luminere 400 watt lamps and humidity was maintained at 80% by an automatically controlled mist humidifier.

2.3. Growth conditions of mutant Brussels sprout plants.

Three week old seedlings of Brussels sprout mutant lines 90/83, 99/83 and 229/83, exhibiting different leaf surface characteristics, (supplied by T.

Hodgkin, S.C.R.I., Dundee) were transferred to 15 cm pots and grown as descibed in 2.1. Herewith the mutants were collectively termed *gemmifera* mutants and cultivar Cambridge No. 5 was designated as the wild type, abbreviated to C5. The genotypes relating to leaf and flower characters are listed below:

90/83 $Go^c/+$, An/.

99/83 gl_3/gl_3, Hr/.

229/83 Fn/Fn, $Go^c/+$, $Hr^1/+$, $gl_6/+$, cp/+, le/le

Go^c	dominant glossy leaf gene.
An	dominant anthocyanin spot on anther tip.
gl_3, gl_6	recessive glossy leaf genes.
Hr, Hr^1	dominant leaf hairs (usually only on first few leaves).
Fn	fern shaped leaf.
cp	crinkly petal.
le	leaf excrescence.

Segregation of alleles resulted in plants which were morphologically glaucous, sub-glaucous or glossy. Glaucous phenotypes were therefore abbreviated to WAX or W, whilst sub-glaucous and glossy phenotypes were abbreviated to INT or I and GLO or G respectively.

2.4. Fungal cultures.

Spores of *Alternaria brassicicola* and *Alternaria alternata* were obtained from cultures of the fungi grown on corn meal agar (CMA) (Oxoid). For each species a single spore culture was derived from isolates on oilseed rape and winter wheat respectively.

Petri-dish cultures were maintained in a Gallenkamp incubator at

temperatures of 20 ± 2 °C and a 12 h cycle of near ultra-violet radiation of intensity 0.47 x 10^{-6} W/cm^2/nm or darkness.

Spore suspensions were prepared by flooding several Petri-dishes with sterile, distilled water and filtering the extract through sterile muslin. This was then centrifuged at 2500 xg for five minutes, washed three times with sterile, distilled water and adjusted to 10^4 spores/ml.

Cultures of *Erysiphe cruciferarum* were maintained on whole plants of the swede cultivar Doon Major under conditions described in 2.1. One day before inoculation plants were shaken to remove spores. New conidia, which had developed overnight were gathered using a dry, sterile paint brush. The paint brush was then shaken above leaf disks, such that conidia were distributed evenly over all disks.

2.5. Scanning Electron Microscopy.

Specimens were prepared in an EMSCOPE SP2000 Cryo-preparation unit and a Cambridge Instruments S250 Scanning electron microscope (SEM).

Sections (10mm x 4mm) were cut from fresh plant material and mounted on copper stubs using Tissue-Tek (Lab-Tek Products, USA). Specimens were frozen rapidly by immersion in sub-cooled nitrogen slush (-210 °C). Frozen specimens were then transferred, under vacuum, to the pre-cooled (-158 °C) evacuated specimen stage of the SEM, where ice crystals were sublimed by heating the stage to a temperature of -70 °C to -60 °C. When the surface was seen to be free of ice, the specimen was taken back to the Cryo-preparation unit where it was sputter coated with gold, then re-

inserted in the cryostage (Jeffree & Read, in press).

Freeze-fractures were carried out using a similar procedure except fracturing took place in the main preparation chamber with a cooled, pointed probe. Specimens were coated immediately.

Examinations were made with the SEM operated at <10kV and a working temperature of -158 °C. Electronmicrographs were taken on Kodak T-Max film using a scan-time of 125 sec (Jeffree & Read, in press).

2.6. Photography

Colour photographs and photomicrographs were taken with an Olympus OM2 camera on Kodacolor (ASA200) daylight slide film and Kodacolor (ASA160) tungsten slide film respectively.

CHAPTER 3

PHYSICAL AND CHEMICAL ATTRIBUTES OF BRASSICA LEAF SURFACES.

3. PHYSICAL AND CHEMICAL ATTRIBUTES OF BRASSICA LEAF SURFACES

3.1. Introduction

The leaves and young stems of all higher plants have a non-living covering termed the cuticle (Fig. 3.1). Cuticles are multi-layered, constructed from materials of diverse chemical composition and physical properties (Jeffree, 1986). The cuticle is composed chiefly of cutin in which are embedded complex mixtures of hydrophobic substances, collectively called wax. Waxy materials are also exuded onto the surface of the plant, where they crystalise to form waxy "blooms". The whole complex is attached onto the epidermis by a pectic material, analogous to and continuous with the intercellular cement of the middle lamella (Martin & Juniper, 1970).

Cuticles develop during the early stages of growth, from precursors synthesised in the epidermal cells and exuded to the surface (Juniper & Jeffree, 1983). Although further deposition depends on species, the general trend is for the structure to thicken, especially on the adaxial surface, as the leaf matures (Martin & Juniper, 1970). Cutin content frequently remains constant but wax deposits decline due to the effects of weathering, friction and other factors. (Denna, 1970; Martin & Juniper, 1970; Wilson, 1984).

The morphology and composition of cuticles appears to be unrelated within species, for plants of different ages and different organs such that no phylogenetic basis can be established (Holloway, 1982a). Moreover since

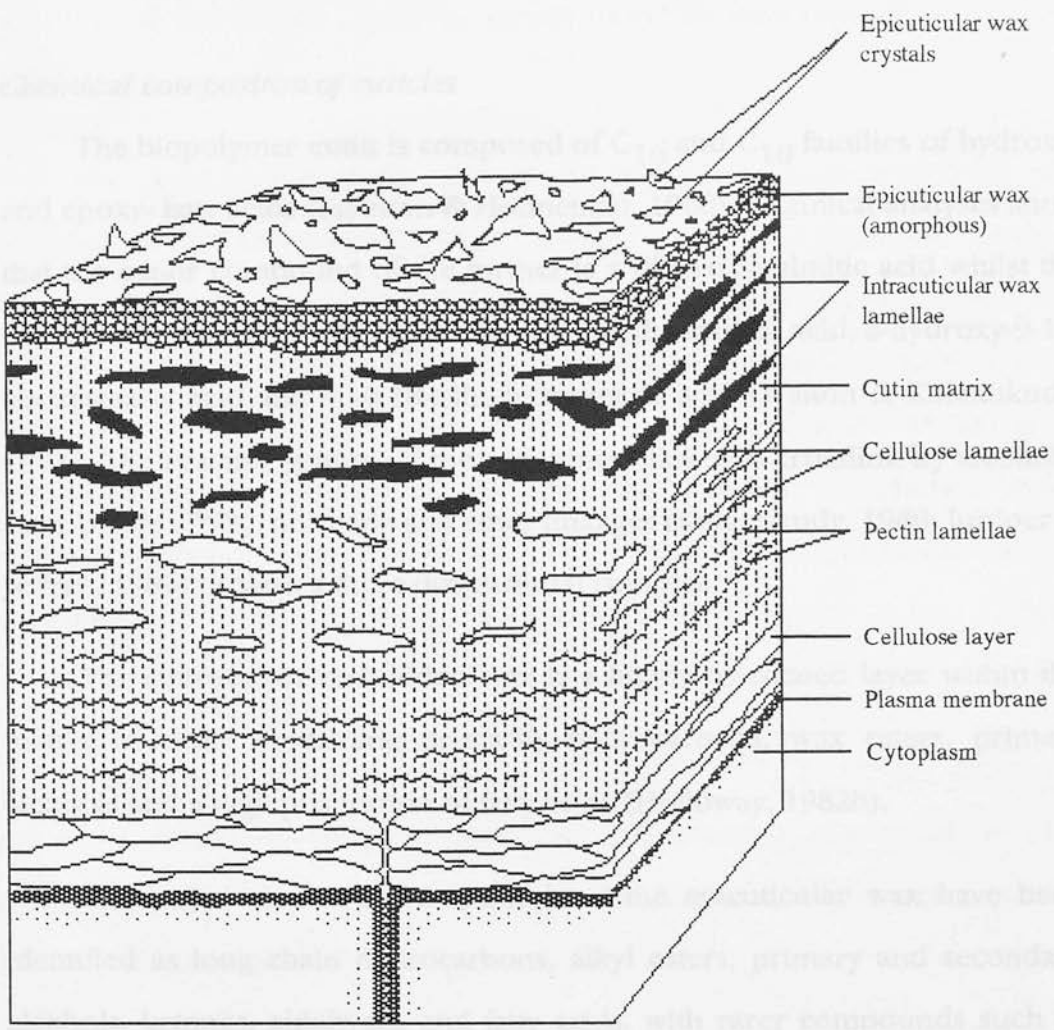


Fig. 3.1. Schematic drawing of plant cuticle.

similar cuticle types are formed on plants from ecologically different backgrounds, then no correlations can be made with ecotype (Holloway, 1982b).

Chemical compositon of cuticles

The biopolymer cutin is composed of C_{16} and C_{18} families of hydroxy- and epoxy- fatty acids (Eglinton & Hunneman, 1968). Chemical analyses show that the major compound of the former is a dihyroxypalmitic acid whilst the major components of the C_{18} family are ω-hydroxyoleic acid, ω-hydroxy-9-10-epoxystearic acid and 9,10,18-trihydroxystearic acid (Walton & Kolattukudy, 1972). The reactive groups of the monomers link and crosslink by alcoholic ester bonds (75%), peroxide and ether linkages (Kolattukudy, 1980; Juniper & Jeffree, 1983) to form a three dimensional complex.

The cuticular or embedded wax is a highly orientated layer within the matrix of cutin, containing primarily hydrocarbons, wax esters, primary alcohols and a high proportion of fatty acids (Holloway, 1982b).

The major classes of compounds of the epicuticular wax have been identified as long chain hydrocarbons, alkyl esters, primary and secondary alcohols, ketones, aldehydes and fatty acids, with rarer compounds such as cyclic constituents and estoloides also present (Baker, 1974; Holloway, Brown & Macey, 1977; Baker, 1982; Jeffree, 1986).

Epicuticular waxes differ both inter- and intra-specifically both qualitatively and quantitatively: the epicuticular wax of blueberry (*Vaccinium ashei*) is mainly composed of β-diketones (Freeman, Albrigo & Biggs, 1979), whereas that of *Brassica* species has alkanes as the dominant class (Baker, 1974; Holloway, Brown & Macey, 1977). Many *Brassica* species display a

great deal of consistency in the chemical composition of their waxes, yet the quantities of individual classes are often peculiar to one species. The C_{29} class predominates in both oilseed rape and Brussels sprout but their proportions are always higher in sprout (90-96%) than rape (82%) (Baker, 1974; Holloway *et al.*, 1977).

Wax chemistry is under genetic control. Single gene mutations can produce salient alterations in the epicuticular wax and therefore the leaf surface, as shown by Macey & Barber (1970) in their study of *Brassica oleracea*. It was suggested an aberration in the C_{30} acid decarboxylation system resulted in diversion of the acid into C_{30} aldehydes. This in turn gave rise to compounds responsible for the glossy character. Additional work (Netting, Macey & Barber, 1972) supported the hypothesis, but the subject was further complicated by the observation that glossy mutants had *anteiso* compounds in the hydrocarbon and fatty acid fractions of their waxes. These compounds were absent in the glaucous normal brassica.

The overall conclusion was that glaucousness depended on an elongation-decarboxylation process and mutations in any of the systems could produce glossy characteristics.

Epicuticular wax morphology

An examination of many species has shown that surface leaf waxes *i.e.* epicuticular waxes, exist in a wide range of morphological forms. The wax layer is of variable thickness, has an underlay of smooth wax, from which may emerge crystalline structures of assorted shapes and sizes. Often these crystals are visible as a "bloom" i.e." the scattering of light due to the waxes having a wavelength similar to that of white light" (Martin & Juniper, 1970). Such surfaces are termed "glaucous", in contrast to the glossy or glabrous form

(Baker, 1974).

The terminology of wax crystal structure is the subject of some controversy, but it is agreed that six basic categories exist: tubes, solid rodlets, filaments, plates, ribbons and granules (Whitecross & Armstrong, 1972; Baker, 1974; Hunt, Holloway & Baker, 1976; Baker, 1982; Jeffree, 1986). Surface waxes often exist as one predominant structural form, but occasionally deposits are composite. Scanning electron microscope studies demonstrate that tube or rod type structures are usually the dominant wax crystal on surfaces which have a pronounced glaucousness (Baker, 1982)

Determination of crystal conformation has been a subject long debated (Whitecross & Armstrong, 1972; Baker, 1974; Jeffree, Baker & Holloway, 1975; 1976; Baker, 1982). Convincing evidence exists to support the view that surface wax structure is strongly dependent on its chemical constitution, although external factors can have major influences (Jeffree *et al.*, 1975; Hunt, Holloway & Baker, 1976, Holloway *et al.*, 1977; Jeffree, 1986). It appears individual components of wax are responsible for particular structures (Jeffree *et al.*, 1975). Thus waxes rich in a certain constituent class will crystalise in one morphological form (Table 3.1).

Tube type waxes tend to be most diverse both structurally and chemically. At least three chemical distinctions are identified, each conferring a discrete morphology: substantial amounts of asymmetrical secondary alcohols result in tubes which are shorter and wider than those whose wax contains high quantities of β-diketones (Jeffree *et al.*, 1975). The third and less frequent form is characterised by having mixtures of symmetrical alcohols and ketones (Holloway, Jeffree & Baker, 1976). Such tubes are extremely long by comparison and have transverse striations visible in

Table 3.1: Relationship between wax constituent and crystalline conformation.

CRYSTALLITE STRUCTURE	MAJOR COMPONENT	SPECIES EXAMPLE
Tubes	Secondary Alcohols Ketones	*Eucalyptus viminalis* *Brassica oleracea* *Picea abies*
Rodlets	Hydrocarbons Secondary Alcohols	*Brassica napus* *Saccharum officinarum*
Filaments	Aldehydes	*Pisum sativum*
Ribbons	Secondary Alcohols Polyestoloides	*Rosa* sp. *Fragaria* sp.
Granules	Primary Alcohols Hydrocarbons	*Citrus limon* *Helianthus annuus*

negatively stained specimens (Jeffree *et al.*, 1976). "Annular ridged" tubes of this nature are the typical wax configuration of *Brassica* species (Baker, 1974; Jeffree *et al.*, 1975; Baker, 1982).

Although wax morphology is principally under chemical control, crystals can be transformed morphologically and, to a lesser extent, chemically by a number of environmental factors. The degree to which the transformation takes place, however, depends on the chemistry of the waxes. Crystals rich in symmetrical alcohols and ketones are extremely sensitive to environmental condition (Jeffree, 1986).

Temperature and light intensity have the most dramatic effects. Effects of changes in light intensity are normally restricted to modifications in the dimensions and density of crystals on the cuticle, whereas changes in temperature subject crystals to reformations of configuration (Baker, 1982). A reduction in radiant energy to 60% resulted in a reduction in dimensions and density of wax rodlets on the surface of *Brassica napus* (Whitecross & Armstrong, 1972). Similar results were found by Baker (1974) in his study of Brussels sprout. Conditions of low light intensity and low temperature produced similar alterations in size and number of wax tubes. A common shift in crystallite conformation was observed in response to a rise in temperature. Upright rods and tubes projecting vertically from the cuticle became laterally growing plates and dendrites (Whitecross & Armstrong, 1972; Baker, 1974).

Functions of the cuticle.

Besides protecting the symplast from invasion by destructive micro-organisms, the cuticle, together with the cell wall, has a fundamental role in the conservation of water, prevention of loss of metabolites due to exudation

and maintaining cellular form. As a result of their chemistry and morphology they have a further role in dictating the architecture and therefore the physico-chemical properties of the leaf surface.

The most important physico-chemical property, with regard to interaction with the external environment, is undoubtedly that of wettability. Wettability is usually measured by the advancing contact angle, defined as "the angle between the surface of a leaf and the tangent plane of a water droplet at the circle of contact between air, liquid and leaf" (Martin & Juniper, 1970). A zero contact angle, although never practically obtained, would indicate a completely wettable surface and any angle up to 180^{o} demonstrates a degree of water repellancy (Fogg, 1947).

Significant elements in determining leaf surface wettability include surface corrugation, hairiness and the chemical and physical nature of the epicuticular wax. The parallel venation system of grasses and the papillose surface of begonia, trap air films which confer a relatively high degree of water repellancy (Martin & Juniper, 1970), whilst the closed patterns of trichomes found on the lower surface of apples and raspberry leaves behave in a similar manner (Holloway, 1971). Epicuticular waxes are often identified as the dominant feature governing wettability in many species. It appears wettability is not a function of the quantity of wax present, rather the form of the wax is the decisive factor. A non-crystalline wax, where platelets are the major configuration, generally allows the surface to be easily wetted. Alternatively a crystalline wax is hydrophobic (Silva-Fernandez, 1965; Troughton & Hall, 1967).

In agricultural practices various substances are applied to leaves. Properties of the cuticle influence the deposition and absorption of pesticides,

herbicides, growth regulators, fertilisers etc. Epicuticular waxes present a problem to the retention of chemicals on the surface, since most applications are water based. To improve the water relations of chemicals and leaf surface, wetting (or surface active) agents are added to spray formulations. Concentration of the wetter and droplet size are significant in efficiency of wetting, which in turn is determined by wetting characteristics of the surface. Ideally the concentration used should be sufficient to enable spread and absorption without allowing complete run off (Hassall, 1982).

Common additives are those chemicals which are termed spreaders. Spreaders act by reducing the surface tension of spray droplets so increasing the extent of the liquid/solid interface. The principle of spreader action is based on the compact stereochemistry of water molecules, a characteristic which confers strong cohesion between the molecules. When spreader molecules are added, they separate preferentially, thrusting the close-packed water molecules apart (Fig. 3.2)(Hassall, 1982). Strong cohesive forces are replaced by weaker adhesive forces, thus the area of contact between droplet and surface is increased.

Both intracuticular and epicuticular waxes influence the permeability of the cuticle. If wax is removed by abrasion then the permeability of the cuticle to water increases up to 500 times (Schönherr, 1976). Denna (1970) found leaves of glossy *Brassica oleracea* lines allowed greater transpiration through the cuticle than glaucous lines.

The chemistry of the cuticle, or of the wax itself, may have inherent physiological significance. Roberts & Martin (1963) claimed that the development of resistance in lime (*Citrus aurantifolia*) to infection by *Gloeosporium limetticola* (= *Glomerella cingulata*) was correlated partly

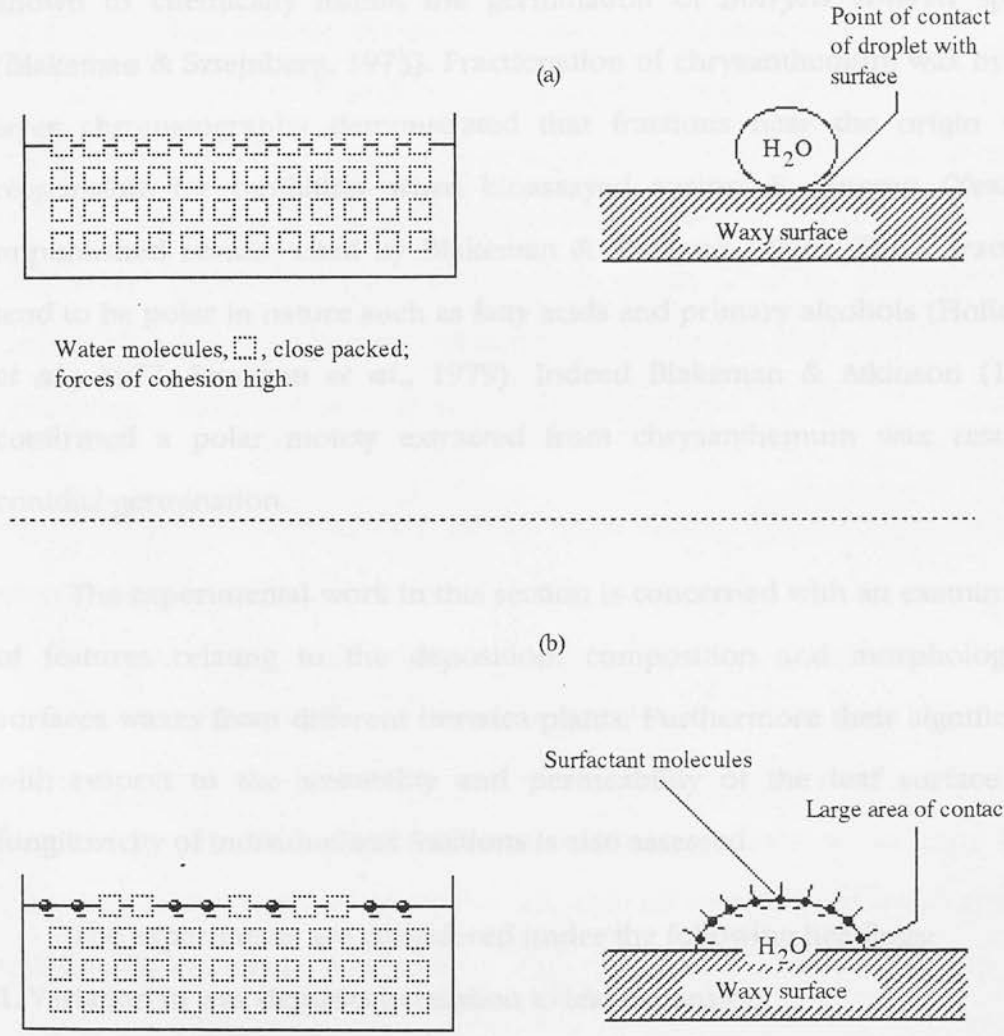


Fig. 3.2. Effect of surfactants on surface tension. (a) Because of high forces of cohesion, there is little contact between droplet and surface in the absence of surfactants. (b) The area of contact increases when lower forces of adhesion replace the high forces of cohesion. (Hassall, 1982).

with increases in cutin acids. Certain waxes have been shown to have antifungal properties. For example the surface wax of beetroot leaves is known to chemically inhibit the germination of *Botrytis cinerea* spores (Blakeman & Sztejnberg, 1973). Fractionation of chrysanthemum wax by thin layer chromatography demonstrated that fractions near the origin were responsible for inhibition when bioassayed against *B. cinerea* (Yeaman, unpublished results; cited by Blakeman & Atkinson, 1976). These fractions tend to be polar in nature such as fatty acids and primary alcohols (Holloway *et al.*, 1977; Freeman *et al.*, 1979). Indeed Blakeman & Atkinson (1976) confirmed a polar moiety extracted from chrysanthemum wax retarded conidial germination.

The experimental work in this section is concerned with an examination of features relating to the deposition, composition and morphology of surfaces waxes from different brassica plants. Furthermore their significance with respect to the wettability and permeability of the leaf surface and fungitoxicity of individual wax fractions is also assessed.

The experiments are considered under the following headings:

1. Variation in wax deposits in relation to leaf expansion.
2. Composition of surface waxes from swede, oilseed rape, Brussels sprout and *gemmifera* mutants.
3. Wax morphology of surface waxes from swede, oilseed rape, Brussels sprout and *gemmifera* mutants.
4. Wettability of leaf surfaces of swede, oilseed rape, Brussels sprout and *gemmifera* mutants.
5. Permeability of leaf surfaces of swede, oilseed rape, Brussels sprout and *gemmifera* mutants.
6. Bioassay of the fungitoxicity of epicuticular wax fractions.

3.2. Materials and methods

3.2.1. Variations in wax deposits in relation to leaf expansion.

Leaves from five 8 week old plants of the three brassica cultivars were briefly immersed (2 seconds) in 75 ml of chloroform to remove wax, then leaf area was determined using an Electroplan belt-driven, photoelectric cell planimeter. The wax extract was filtered, waxes freed by evaporation of solvent in a fume cupboard, and expressed as weight per unit area (Silva-Fernandez, 1965; Rawlinson, *et al.*, 1978).

3.2.2. Composition of surface waxes from swede, oilseed rape, Brussels sprout and **gemmifera** *mutants*

Isolated epicuticular waxes, as above, from apical, middle and basal leaves of the three brassica cultivars together with waxes from the middle leaves of the *gemmifera* mutants, were examined by Preparative Layer Chromatography (P.L.C.). P.L.C. was used in preference to thin layer chromatography because a greater volume of sample could be applied, enabling finer clarity of components.

Wax samples were dissolved in distilled chloroform and applied (100 mg/ml (w/v)) to 1 mm layers of silica gel G using distilled xylene as running solvent. Spots were detected by exposing the dried plates to iodine vapour in sealed chambers (Baker, 1974; Baker Procopiou & Hunt, 1975), and identified by comparison with commercial standards or various biochemical tests (Appendix 3.1).

Quantitative analyses of wax were made using a Pye Unicam GCD Gas-Liquid Chromatograph fitted with flame ionisers and Trivector TRIO

Chromatography Computing Integrater. Constituent classes were separated on a 1 x 2 mm glass column packed with 10% OV3 on 80-100 mesh Chromosorb WHP (J. Hands, *pers comm*). Temperature was programmed at 250-300 ^{o}C with a rise rate of 2 ^{o}C/min and nitrogen flow rate of 45 ml/min (Tulloch, 1973; Baker *et al.*, 1975).

Retention times of hydrocarbons, primary alcohols and fatty acids were determined using commercial reference compounds (n-nonacosane, hexacosanol and lignoceric acid respectively, Sigma, Poole) The remaining classes were determined by comparison with isolated fractions (Baker *et al.*, 1975; Freeman *et al.*, 1979). Amounts of compounds were calculated from their response relative to n-tetracosane (Baker, 1974; Holloway *et al.*, 1977).

3.2.3. Wax morphology of surface waxes from swede, oilseed rape, Brussels sprout and **gemmifera** *mutants.*

Surfaces of apical, middle and basal leaves of plants from the three brassicas grown in glasshouse conditions together with mid-leaves from further plants grown under various environmental conditions with respect to temperature and light intensity (Chapter 2.2), were examined by scanning electron microscopy. In addition, mid-leaves from *gemmifera* mutants were prepared for study. Specimen preparation and examination were as described in general materials and methods and wax crystal configuration was considered.

As indicated in the introduction, classification of wax morphology is partly subjective and, hence, the use of terms in the present work has been approached with caution. Rectangular structures projecting from the surface were designated rodlets. The term tube has been avoided, as this implied a hollow structure: techniques employed did not allow such detail to be

determined. Waxes with a rippled appearance were termed plates (C.E. Jeffree, *pers comm*).

3.2.4. Wettability tests of leaf surface of swede, oilseed rape, Brussels sprout and **gemmifera** *mutants.*

A series of dilutions (0.001%-0.01%) of the surfactant Triton-X-100 (BDH, Poole) was prepared in distilled water. Assessment of the affinity of the leaf surface for water was accomplished by allowing a droplet of each solution to form at the end of a micropipette and lowering until the droplet touched the plant surface in a horizontal position (Fig. 3.3). The leaf was then held vertically and the running behaviour of the droplet was observed. The concentration of surfactant which permitted the water droplet to lose its spherical shape and create a non-contracting trail when the liquid ran off the leaf surface (Fig 3.4) was assigned as the concentration which effected wetting (Silva-Fernandez, 1965; Rawlinson *et al.*, 1978). Experiments to investigate factors governing wettability using this method were as follows:

a. In a preliminary study all leaves from five plants of the three brassica cultivars were tested to examine water relationships in relation to leaf position.

b. Two hours previous to analysis five plants of each brassica were sprayed with the surfactant Agral at a working concentration of 0.3 ml/l. Six leaves from positions A-F, where A leaves were apical and F leaves were basal, were taken from each of these treated plants together with 6 leaves from each of five untreated brassicas. The latter were the controls. The design was a split-split plot design with surfactant treatment as main plot, brassica plant as sub-plot and leaf position as sub-sub-plot.

Fig. 3.3. Water droplet on middle leaf of swede

Fig. 3.4 Effective wetting concentration of Triton- X -100 creating a non- contracting trail on middle leaf of oilseed rape.

Fig. 3.3.

Fig. 3.4.

c. Six leaves from positions termed A-F as before, were taken from each of five plants of the three brassica cultivars which had been raised under different environmental conditions of temperature and light intensity. The design was in the form of a split-split-split plot with environmental treatment as main plot, brassica plant as sub-plot and leaf position as sub-sub-plot.

d. Six leaves similar to the previous experiment were removed from each of five plants of the *gemmifera* mutants and the wild type. The design was a split-plot design with mutant family as main plot and leaf position as sub-plot.

e. Measurement of contact angles.

In order to validate the previous method as an accurate technique for measuring wettability, records were made of the contact angles of water droplets on the leaf surfaces of the three brassicas, comparable to those used in experiment (a). Water droplets applied to the surface by a micropipette were photographed on Kodak ASA125 black and white film. Processed negatives were magnified and projected onto a screen where the contact angle was measured (Fogg ,1947; Troughton & Hall, 1968). Contact angles of all leaves from four swede, oilseed rape and Brussels sprout plants were assessed.

3.2.5. Permeability of leaf surfaces of swede, oilseed rape and Brussels sprout and **gemmifera** *mutants.*

Permeability of leaf surfaces was assessed by measuring the conductivity of a 15 μl water droplet, pipetted onto the surface. A microelectrode connected to a WPA CMD400 digital conductivity meter on the 10^{-6} siemens scale, was dipped into the droplet 0, 2, 4, 6, 8, 10, 18 and 24 hours post-application. Conductivity readings were recorded on leaves in two experiments:

a. Disks (14 mm diameter) of leaves from positions assigned A-F as before were removed from each of ten brassica plants. One leaf disk from each position of each test plant (i.e. 18 disks) was placed at random in a square compartmentalised Petri-dish containing water agar with 8 ppm benzimidazole added to delay senescence, herewith referred to as SQPs. The experiment was replicated ten times to give a total of ten Petri dishes containing 180 disks. Results were analysed statistically for initial conductivity and changes over 10 and 24 hours. A spit-plot design was used with brassica plants forming main plots and leaf positions sub-plots.

b. Ten leaf disks (14 mm diam) of apical, middle and basal leaves were taken from each of the seven *gemmifera* mutants and wild type. The same procedure as above was followed using ten replicates to give ten Petri-dishes containing 240 disks and with a split-plot design with *gemmifera* mutant as the main plot. Results were analysed as before for readings at 0, 10 and 24 hours after applying drops.

3.2.6. Bioassay of the fungitoxicity of epicuticular wax fractions.

One P.L.C. plate run for brassica plant and leaf position was sprayed using an atomiser with 100 ml of a spore suspension of *Cladosporium* sp., prepared as described for *Alternaria* (Chapter 2.4), exchanging sterile, distilled water in the final spore suspension for Czapex Dox liquid medium. The final concentration of spores was 10^6 spores/ml. The plate was placed in a sealed, moist chamber and incubated in a Gallenkamp incubator at 20°C with a 12 hour daylength. After 5 days the plate was examined for zones of inhibition.

3.3. Results

3.3.1. Variation in wax deposits in relation to leaf expansion.

Changes in leaf surface area and wax deposits with leaf position are shown in Figs 3.5 and 3.6 respectively. Leaf area increased only slightly with descending leaf position in the case of young leaves (*i.e.* those at apical positions) for all three brassica cultivars. At the middle leaf positions, leaves of swede, compared with other brassicas, showed a more rapid expansion with age, up to the middle leaf where maximum size was almost reached. Leaf expansion for oilseed rape and Brussels sprout, on the other hand, with one exception, followed a more steady, continuous pattern, approaching maximum leaf size in basal leaf positions.

Variations in quantities of wax between and within brassicas were distinct in apical leaf postions, but were otherwise minimal (Fig 3.6). Wax levels of Brussels sprout and oilseed rape were maximum in the youngest, uppermost leaf and declined sharply towards middle positions; thereafter little decrease was recorded. Levels of wax on swede followed similar trends, except that maximum levels were recorded in the sub-apical leaf.

Fig. 3.5. Leaf area of different brassicas in relation to leaf position

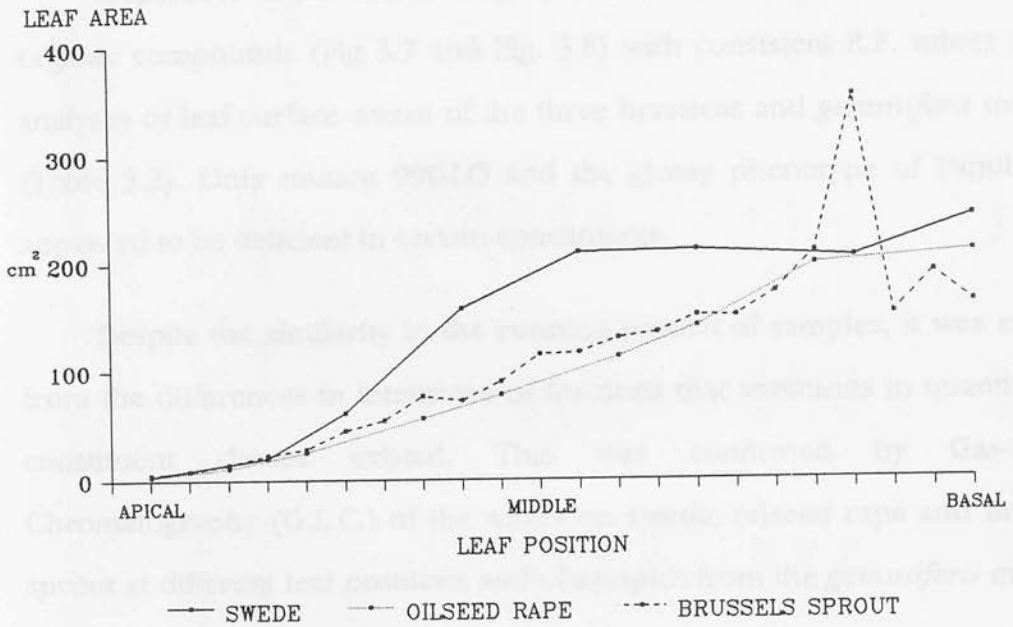
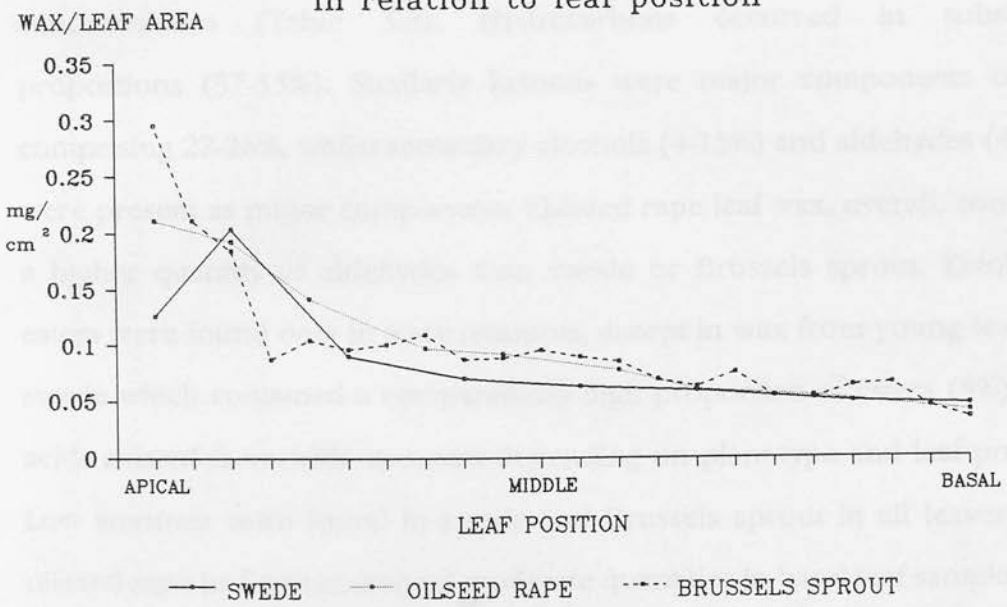


Fig. 3.6. Amounts of epicuticular waxes per unit leaf area of different brassicas in relation to leaf position



3.3.2. Composition of surface waxes from swede, oilseed rape, Brussels sprout and **gemmifera** *mutants.*

Preparative Layer Chromatography (P.L.C.) detected eight classes of organic compounds (Fig 3.7 and Fig. 3.8) with consistent R.F. values in the analyses of leaf surface waxes of the three brassicas and *gemmifera* mutants (Table 3.2). Only mutant 99GLO and the glossy phenotype of Family 229 appeared to be deficient in certain constituents.

Despite the similarity in the running pattern of samples, it was evident from the differences in intensities of fractions that variations in quantities of constituent classes existed. This was confirmed by Gas-Liquid Chromatography (G.L.C.) of the waxes on swede, oilseed rape and Brussels sprout at different leaf positions and of samples from the *gemmifera* mutants (Tables 3.3 and 3.4).

Analyses of whole wax revealed the presence of the eight separate components in all samples of the three brassica cultivars but in varying concentrations (Table 3.3). Hydrocarbons occurred in substantial proportions (37-55%). Similarly ketones were major components of wax comprising 22-26%, whilst secondary alcohols (4-15%) and aldehydes (4-14%) were present as minor components. Oilseed rape leaf wax, overall, contained a higher quantity of aldehydes than swede or Brussels sprout. Ketols and esters were found only in trace amounts, except in wax from young leaves of swede which contained a comparatively high proportion of esters (5%). Fatty acids existed in variable amounts depending on plant type and leaf position. Low amounts were found in swede and Brussels sprout in all leaves while oilseed rape leaf wax contained moderate quantities in basal leaf samples.

Ontogenic changes in wax were most pronounced in the secondary

Table 3.2. R.F. values of wax samples from different brassicas and different leaf positions and gemmifera mutants analysed by Preparative Layer Chromatography.

	Spots (in order of elution)							
	Fatty acids	1^{o} alcohols	Ketols	2^{o} alcohols	Aldehydes	Ketones	Esters	Hydro-carbons
BRASSICA/ LEAF POSITION								
Swede								
apical	0.03	0.09	0.22	0.32	0.61	0.77	0.91	0.97
middle	0.04	0.12	0.26	0.34	0.59	0.75	0.92	0.99
basal	0.04	0.13	0.24	0.35	0.60	0.74	0.88	0.98
Oilseed rape								
apical	0.03	0.11	0.26	0.35	0.60	0.78	0.91	0.98
middle	0.04	0.13	0.24	0.36	0.60	0.77	0.92	0.98
basal	0.03	0.12	0.24	0.36	0.61	0.76	0.89	0.98
Brussels sprout								
apical	0.03	0.12	0.26	0.34	0.60	0.78	0.90	0.98
middle	0.03	0.12	0.23	0.35	0.60	0.77	0.92	0.98
basal	0.04	0.13	0.24	0.36	0.61	0.77	0.90	0.98

Table 3.2. continued.

	Fatty acids	1^{o} alcohols	Ketols	2^{o} alcohols	Aldehydes	Ketones	Esters	Hydro-carbons
MUTANT LINE								
C5	0.03	0.12	0.24	0.34	0.56	0.68	0.81	0.98
90W	0.04	0.11	0.23	0.35	0.55	0.69	0.82	0.98
90I	0.04	0.11	0.24	0.34	0.58	0.69	0.82	0.98
90G	0.03	0.12	0.25	0.38	0.58	0.69	0.84	0.99
99G	0.03	0.11	*	*	*	*	0.82	0.99
229W	0.04	0.11	0.25	0.34	0.56	0.69	0.84	0.99
229I	0.03	0.11	0.25	0.34	0.56	0.68	0.84	0.98
229G	0.03	0.11	0.23	*	0.57	*	0.85	0.99

* - FRACTIONS NOT DETECTED

Fig. 3.7 Preparative Layer Chromatography of brassica epicuticular waxes. From left to right- apical leaves of swede, oilseed rape and Brussels sprout, middle leaves of swede, oilseed rape and Brussels sprout and basal leaves of swede, oilseed rape and Brussels sprout.

Fig 3.8. Preparative Layer Chromatography of *gemmifera* mutants. From left to right- wild type, 99GLO, 90WAX, 90IN, 90GLO, 229WAX, 229IN, 229GLO.

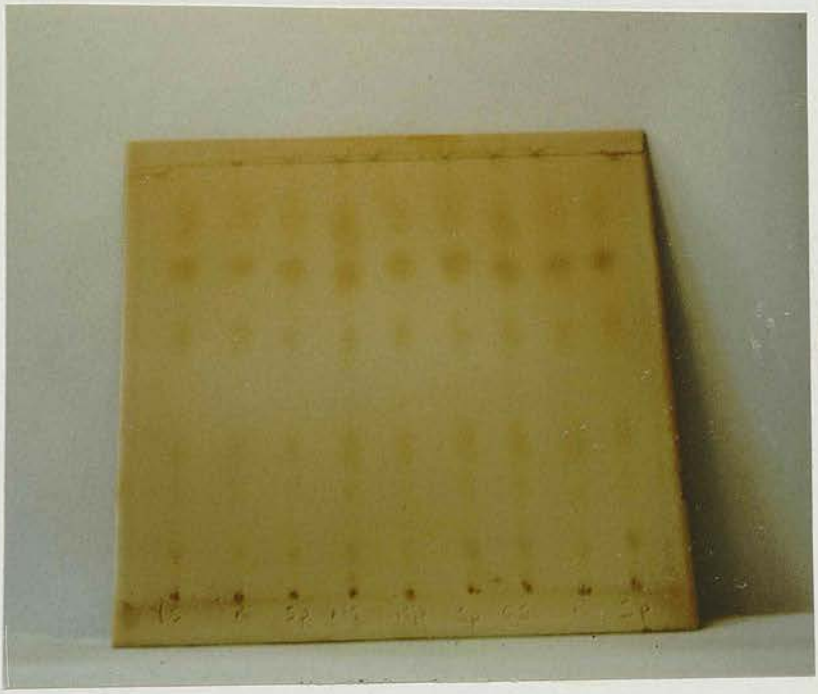


Fig. 3.7.

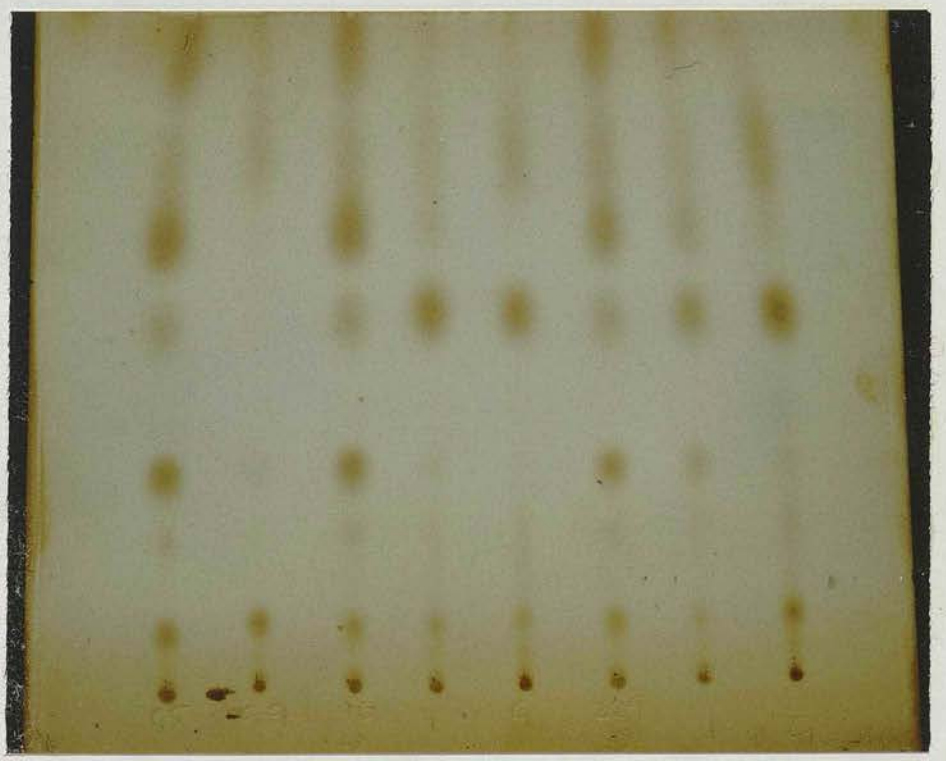


Fig. 3.8.

TABLE 3.3. GLC of apical(A), mid(M), and basal(B) leaf waxes from different brassicas

Peaks in order of elution by P.L.C.	Percentage total wax								
	Swede			Oilseed rape			Brussels sprout		
	A	M	B	A	M	B	A	M	B
Fatty Acids	2	3	3	1	2	8	4	3	2
1° Alcohols	4	8	6	4	5	5	5	5	9
Ketols	1	1	1	1	1	0.5	1	1	1
2° Alcohols	4	10	11	3	8	15	5	14	11
Aldehydes	7	4	10	14	12	13	6	6	8
Ketones	26	23	22	23	24	21	22	22	24
Esters	5	1	2	2	1	0.5	2	2	2
Hydrocarbons	51	50	45	52	47	37	55	47	43

TABLE 3.4. GLC of gemmifera mutants showing different leaf surface features (sampled from mid-leaves)

Peaks in order of elution by P.L.C.	Percentage total wax							
	C 5	9 0			9 9	2 2 9		
	(Wild type)	(waxy)	(intermediate)	(glossy)	(glossy)	(waxy)	(intermediate)	(glossy)
Fatty Acids	3	3	2	2	0.5	2	4	3
1° Alcohols	5	4	12	6	73	4	6	4
Ketols	1	1	1	1	0.5	0.5	1	2
2° Alcohols	14	13	10	5	7	13	7	4
Aldehydes	6	3	57	73	0	0.5	11	75
Ketones	22	24	7	4	2	21	19	3
Esters	2	2	1	2	15	2	2	3
Hydrocarbons	47	50	11	7	2	57	50	6

alcohol and hydrocarbon fractions of all three brassicas. Secondary alcohols were present at lower levels in leaf wax as leaves emerged, whilst the percentage of hydrocarbons decreased as leaves aged.

The composition of waxes from *gemmifera* mutants is given in Table 3.4. Waxes from the wild type and the waxy phenotypes of mutant Families 90 and 229 were remarkably alike, being notably high in quantities of secondary alcohols, ketones and especially hydrocarbons. These leaves also had a common glaucous appearance (Figs 3.9, 3.10 and 3.11). In comparison, leaves with a glossy character, i.e. the glossy phenotypes of 90 (Fig. 3.10) and 229 (Fig.3.11), also had similar wax chemistry distinguished by the abundance of aldehydes (73-75%) and reductions in the hydrocarbon fraction.

Waxes from the intermediate phenotypes tended to show a composition in keeping with their appearance. The intermediate type of 90, which most closely resembled the glossy phenotype in appearance, had a similar wax profile, but with increased levels of secondary alcohols, related to the waxy phenotype. Ketones and hydrocarbons were the dominant constituents of 229 intermediate allied with the waxy phenotype, which it most resembled. However, low levels of secondary alcohols were present as in the glossy phenotype.

Mutant 99GLO appeared to have an independent wax profile. Despite having a glossy characteristic (Fig. 3.9) no aldehydes were detected, and primary alcohols comprised the major component (73%). At 15%, the ester fraction was an additional unusual feature of this wax, compared with trace amounts found in other waxes.

Fig. 3.9. Mid-leaves of wild type (left) and 99GLO.

Fig. 3.10. Mid leaves of 90WAX (left), 90IN (centre) and 90GLO (right).

Fig. 3.11. Mid-leaves of 229WAX (right), 229IN (centre) and 229GLO (right).

3.3.3. Wax morphology of surface waxes from swede, oilseed rape, Brussels sprout and **gemmifera** *mutants.*

a. Wax morphology in relation to brassica and leaf position.

Brassica leaf surfaces were overlaid by a film of crystalline wax as illustrated in Fig 3.12. Rodlet-like structures were the dominant conformation interspersed with regions of amorphous wax, the extent of these areas varying with different brassica plants.

On leaves of swede the amorphous wax was most extensive and had a distinct rippled appearance resembling plates, which were enclosed by rodlets. A more even distribution of greater density of rods was exhibited on leaves of oilseed rape and Brussels sprout, such that more of the cuticle surface was obscured. Waxes were crystalline on all leaves including those which were just emerging. Size (Table 3.5) and, to some extent, distribution were similar in juvenile and middle leaves of oilseed rape and Brussels sprout. However decreases in size and density of projections were observed at the onset of senescence. Crystals became degraded and large areas of the cuticle surface were exposed on the basal leaves (Fig 3.12). With swede, wax development was complete shortly after leaf emergence, since apical leaves carried a heavy deposit of large crystals, whilst smaller crystals sparsely distributed were observed on middle and basal leaves (Fig. 3.12; Table 3.5).

Table 3.5. Dimensions of epicuticular wax crystals on the leaf surfaces of brassicas in relation to leaf position.

		Leaf Position		
		apical	middle	basal
Brassica type				
SWEDE	length(μm)	4.18	2.02	2.44
	width (μm)	0.81	0.55	0.69
OILSEED RAPE		4.67	4.00	3.04
		0.62	0.50	0.45
BRUSSELS SPROUT		4.02	3.67	0.95
		0.65	0.53	0.71

Table 3.6. Dimensions of epicuticular wax crystals on brassicas grown in different enviroments.

		Environmental condition			
		CT/LL	CT/HL	WT/LL	WT/HL
Brassica type					
SWEDE	length(μm)	1.74	2.30	2.47	4.87
	width (μm)	0.47	0.54	0.35	0.45
OILSEED RAPE		1.29	2.88	3.22	4.56
		0.55	0.46	0.52	0.40
BRUSSELS SPROUT		2.02	3.82	3.06	4.76
		0.58	0.49	0.42	0.42

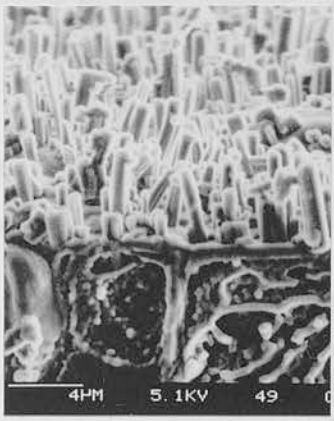
CT- cool temperature (12°C)
WT- warm temperature (18°C)
LL- low light intensity (4500 lux)
HL- high light intensity (9000 lux)

Fig. 3.12 (opposite). SEMs of freeze-fractures of brassica leaf surfaces showing epicuticular wax crystals. From left to right- apical, middle and basal leaves.

Top row: Swede.

Middle row: Oilseed rape.

Bottom row: Brussels sprout.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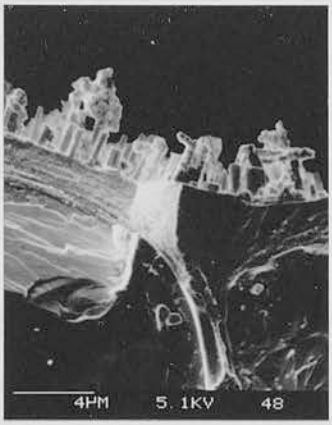
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4μM 5.1KV 49


4μM 5.1KV 49


4μM 5.1KV 49


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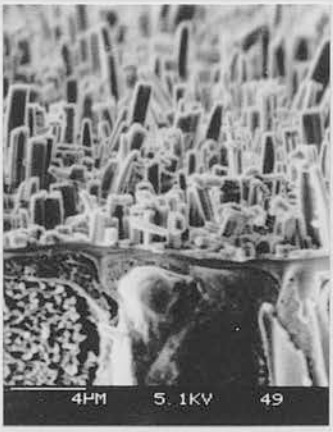

4μM 5.1KV 49


4μM 5.1KV 49


4μM 5.1KV 49

b.Environmental alterations to wax morphology in relation to brassica, temperature and light intensity.

Configuration, size and distribution of the crystalline waxes were significantly modified by the various environmental conditions (Fig 3.13; Table 3.6).

The morphology of the wax was clearly affected by temperature. Low temperatures produced rodlet-like waxes which projected upright from the surface. At the higher temperature waxes consisted of a composite of rods and dendrites. The latter, formed at the top of the rods as branches, were such that the surface was effectively covered. These effects were evident in all three brassicas irrespective of light intensity.

Effects of light intensity were most evident under the lower temperature regime and were restricted to alterations in size and distribution of crystals (Table 3.6; Fig. 3.13). The observation was particularly well illustrated in swede leaves. Under conditions of low light intensity and low temperature crystals were short (1-2μm) and distributed sparsely, indicating low crystallisation rates for waxes. Increasing light intensity resulted in an increase in length (2-4μm) and density of crystals. Although corresponding changes in the dimensions of crystals were recorded at the higher temperature regimes, differences in density were more difficult to interpret due to the complex network of dendrites, but were assumed to follow the same trend.

c. Wax morphology of different **gemmifera** *mutants.*

Pronounced differences in wax morphology occurred on *gemmifera* mutants (Fig 3.14). The waxy phenotypes of 90 and 229 mutant lines had heavy deposits of wax similar in configuration and density to the wild type (C5), whereas the glossy phenotypes had a smooth, amorphous layer (229) or

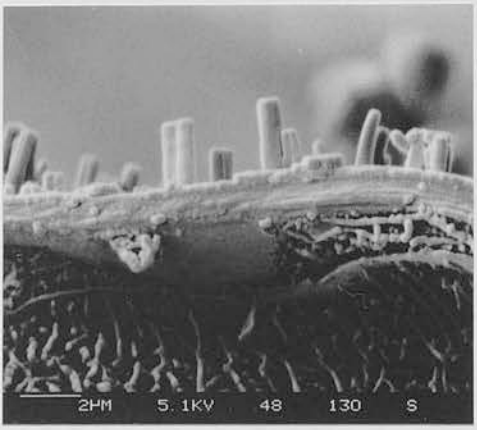
Fig. 3.13a. (opposite). SEMs of freeze-fractures of surfaces of swede mid-leaves grown in different environmental conditions showing epicuticular wax crystals.

Top left: Low temperature, low light intensity.

Top right: Low temperature, high light intensity.

Bottom left: High temperature, low light intensity.

Bottom right: High temperature, high light intensity.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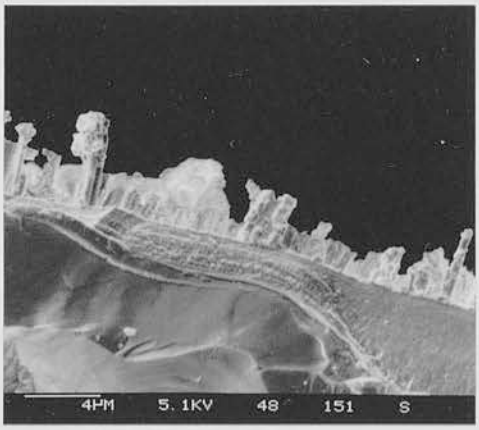
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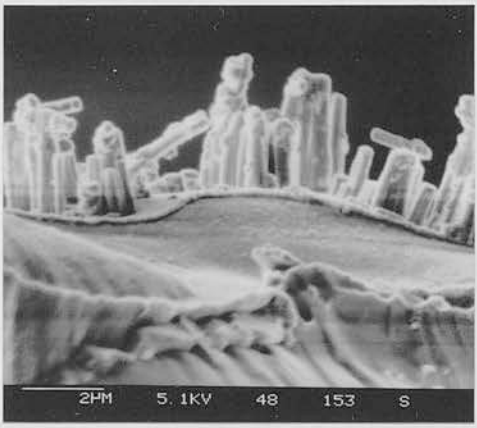
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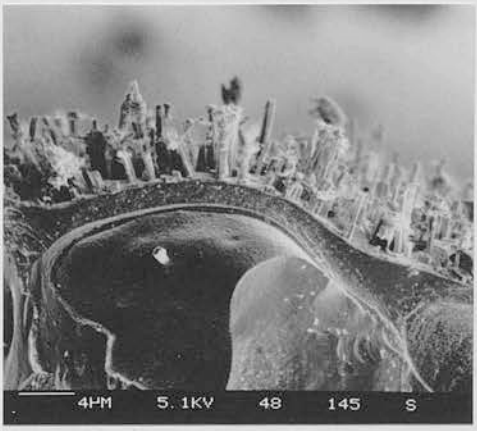

4μM 5.1KV 48 145 S

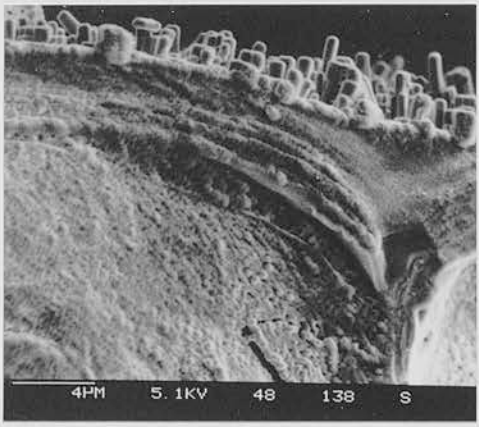
Fig. 3.13b. (opposite). SEMs of freeze-fractures of surfaces of oilseed rape mid-leaves grown in different environmental conditions showing epicuticular wax crystals.

Top left: Low temperature, low light intensity.

Top right: Low temperature, high light intensity.

Bottom left: High temperature, high, low intensity.

Bottom right: High temperature, high light intensity.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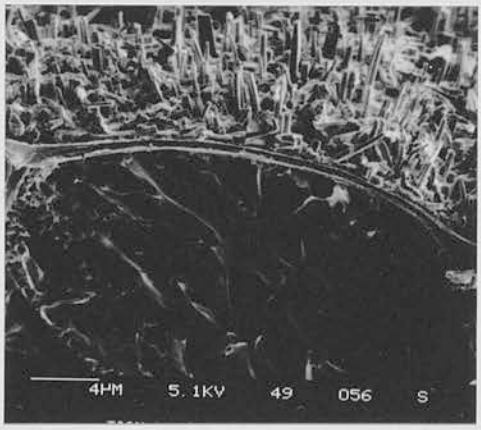
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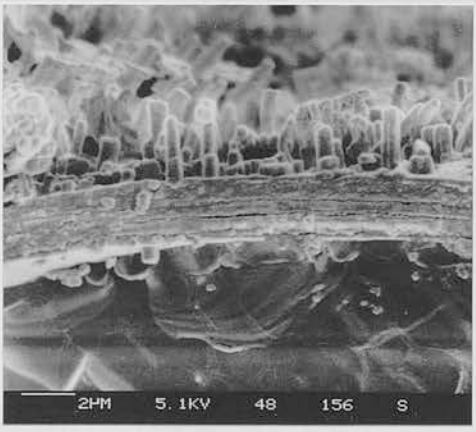
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4μM 5.1KV 49 043 S

Fig. 3.13c. (opposite). SEMs of freeze-fractures of surfaces of Brussels sprout mid-leaves grown in different environmental conditions showing epicuticular wax crystals.

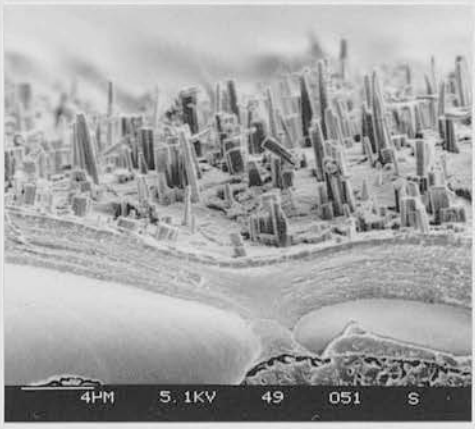
Top left: Low temperature, low light intensity.

Top right: Low temperature, high light intensity.

Bottom left: High temperature, low light intensity.

Bottom right: High temperature, high light intensity.


4μM 5.1KV 48 137 S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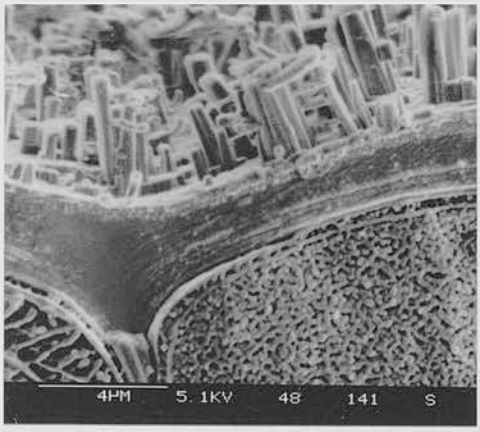
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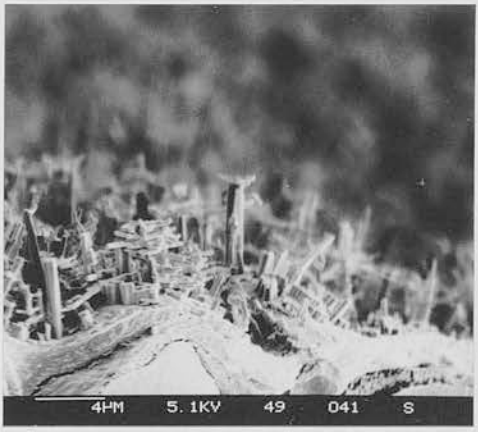

4μM 5.1KV 49 041 S

a continuous film with occasional crystalline structures (90). The intermediate phenotypes mirrored either the waxy phenotype (229) or the glossy phenotype (90). Mutant line 99GLO had a distinctive wax character, with clusters of small plate like structures within a smooth, otherwise amorphous wax.

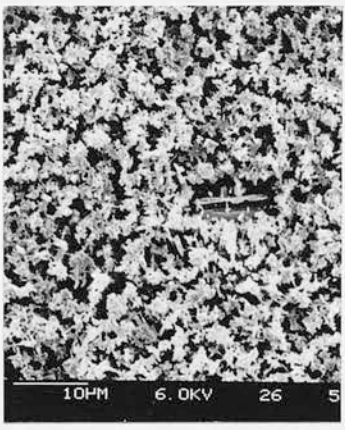
Fig. 3.14 (opposite). SEMs of *gemmifera mutant* leaf surfaces showing epicuticular wax crystals.

Top row: left to right- C5, 90WAX, 229WAX.

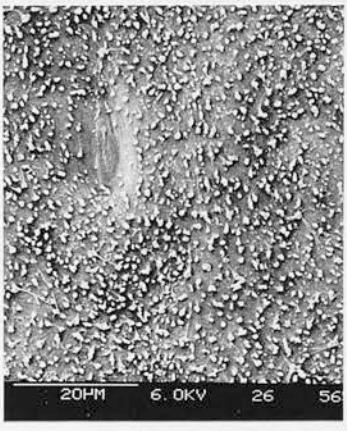
Middle row: leaf to right- 90IN, 229In.

Bottom row: left to right- 99GLO, 90GLO, 229GLO.


4UM 6.0KV 26 63


10UM 6.0KV 26 5


10UM 6.0KV 26 74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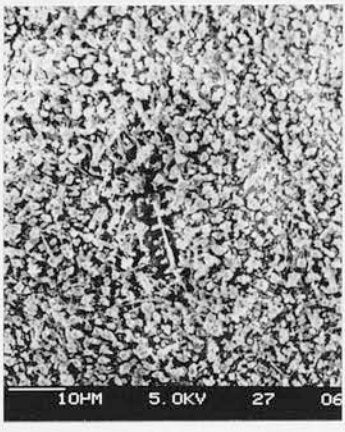
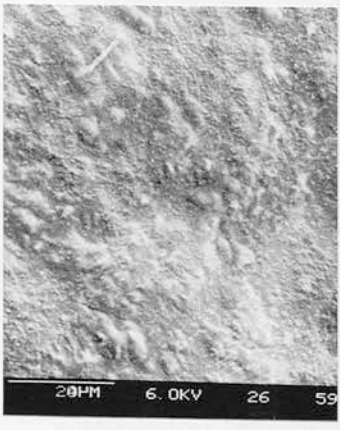

10UM 5.0KV 27 06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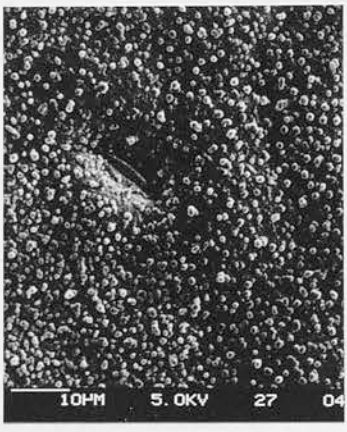

10UM 5.0KV 27 04


10UM 6.0KV 26 7

3.3.4. Wettability of leaf surfaces of swede, oilseed rape, Brussels sprout and **gemmifera** *mutants.*

a.Wettability in relation to brassica type and leaf postion.

The leaf surface water relations of swede, oilseed rape and Brussels sprout changed as leaves developed (Fig 3.15). Low concentrations of surfactant were required to wet emergent leaves indicating a low degree of water repellancy. Water repellancy increased to a maximum level in mid-leaf positions then decreased rapidly with increasing leaf age. Leaves at the onset of senescence had similar wettabilities as apical leaves, indicated by an equivalent concentration of surfactant required to effect wetting.

The ability to repel water from the surface was positively correlated with the presence of a waxy "bloom". Young leaves, although waxy, did not have an obvious "bloom" and allowed water droplets to spread over a large area. The opposite was observed for the middle leaves which carried a heavy "bloom".

Different brassicas varied in the wettability properties of their leaves. Apical leaves of Brussels sprout had a higher affinity for water compared with swede and oilseed rape. However by the mid stages of leaf development water repellancies had reversed, such that an increase in surfactant concentration was required to wet Brussels sprout leaves above that necessary for swede and oilseed rape. As leaves of all three brassicas aged, and water repellancies declined, the wetting concentration of surfactant approached almost similar levels, but Brussels sprout remained most water repellant.

Calculation of contact angles confirmed that the effective surfactant concentration to wet leaves was an indicator of wettability. Therefore the procedure was confined to this first experiment. Contact angle measurements

Fig. 3.15. Concentration of surfactant required to effect wetting of leaves of different brassicas in relation to leaf position

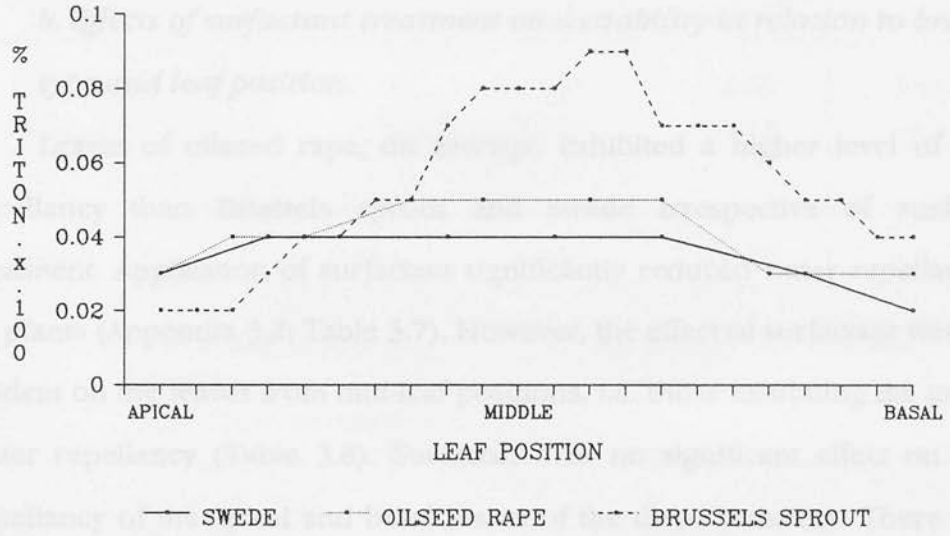
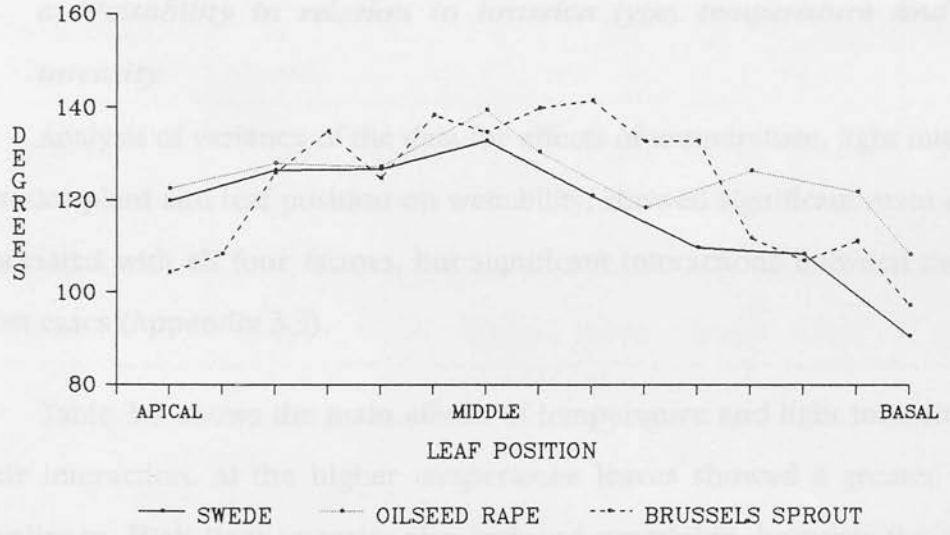


Fig. 3.16. Contact angles of leaves of different brassicas in relation to leaf position



followed similar patterns (Fig 3.16), with regards to leaf position as determinations with surfactant, although they did not discriminate as clearly between different brassica types at mid- and basal leaf positions.

b. Effects of surfactant treatment on wettability in relation to brassica type and leaf position.

Leaves of oilseed rape, on average, exhibited a higher level of water repellancy than Brussels sprout and swede irrespective of surfactant treatment. Application of surfactant significantly reduced water repellancy in all plants (Appendix 3.2; Table 3.7). However, the effect of surfactant was most evident on the leaves from mid-leaf positions, i.e. those exhibiting the greatest water repellancy (Table 3.8). Surfactant had no significant effect on water repellancy of the apical and basal leaves of the three brassicas. There was a significant interaction between Brassica plant and leaf position, with the apical leaves of oilseed rape proving more water repellant than other brassicas (Fig. 3.17).

c. Wettability in relation to brassica type, temperature and light intensity.

Analysis of variance of the data for effects of temperature, light intensity, brassica plant and leaf position on wettability, showed significant main effects associated with all four factors, but significant interactions between them in most cases (Appendix 3.3).

Table 3.9 shows the main effects of temperature and light intensity and their interaction. At the higher temperature leaves showed a greater water repellancy. High light intensity also reduced wettability, however the effects were restricted to the higher temperature.

Table 3.7. Concentration of surfactant (%), required to effect wetting of leaves of different brassicas in relation to treatment with Agral (average of leaf position).

SURFACTANT TREATMENT	BRASSICA TYPE Swede	Oilseed rape	Brussels sprout	Mean
Untreated	0.042	0.051	0.046	0.046
Treated	0.029	0.043	0.034	0.035
Mean	0.036	0.047	0.040	0.041

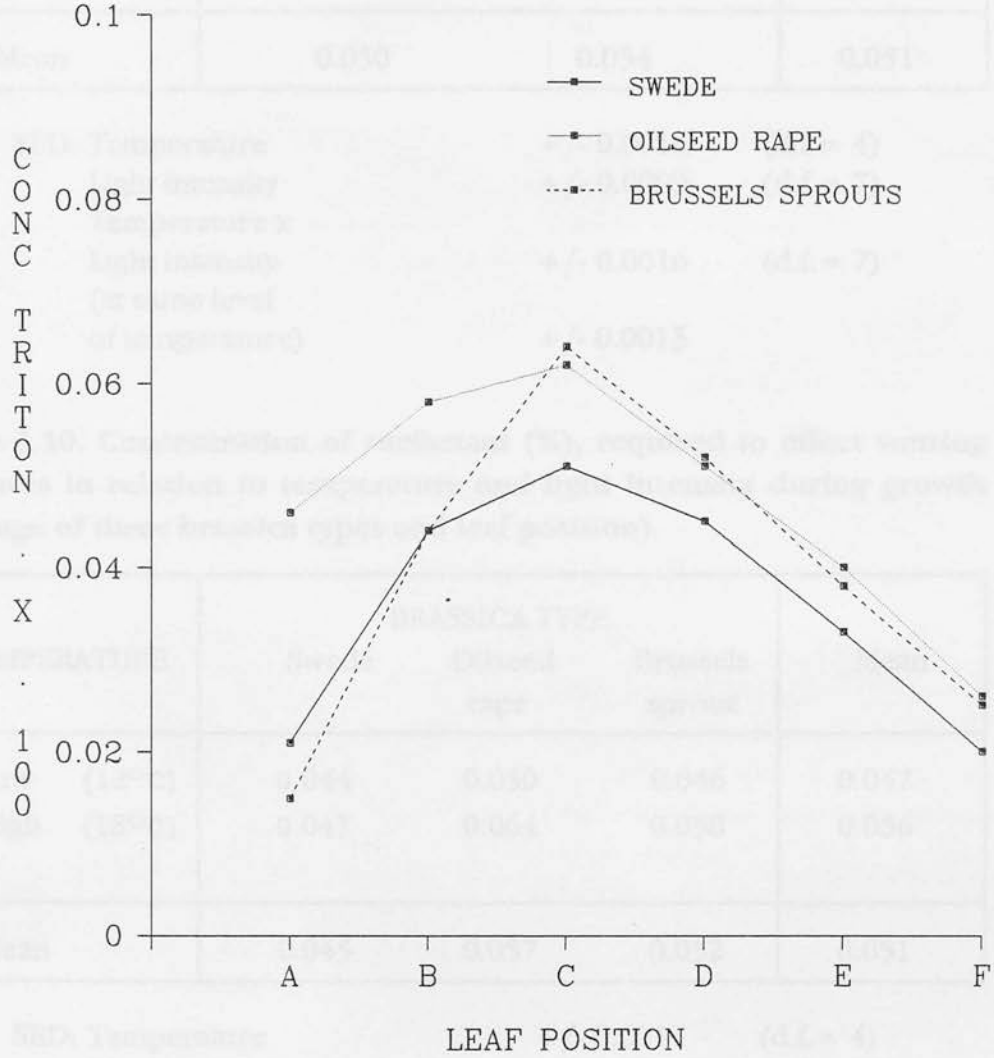
SED: Surfactant treatment	+/- 0.0013	(d.f. = 4)
Brassica type	+/- 0.0018	(d.f. = 16
Surfactant treatment x Brassica type (at same level of surfactant)	+/- 0.0025 +/- 0.0026	(d.f. = 16)

Table 3.8. Concentration of surfactant (%), required to effect wetting of brassica leaves from different leaf positions in relation to treatment with Agral (average of three brassicas).

SURFACTANT TREATMENT	LEAF POSITION A (Apical)	B	C	D	E	F	Mean
Untreated	0.028	0.054	0.071	0.060	0.042	0.022	0.046
Treated	0.026	0.043	0.047	0.039	0.032	0.025	0.035
Mean	0.027	0.049	0.059	0.049	0.037	0.024	0.041

SED: Surfactant treatment	+/- 0.0013	(d.f. = 4)
Leaf position	+/- 0.0035	(d.f. = 116)
Surfactant treatment x Leaf position (at same level of surfactant)	+/- 0.0047 +/- 0.0049	(d.f. = 116)

Fig. 3.17. Concentration of surfactant required to effect wetting of brassica leaves in relation to leaf position (averaged for surfactant treatment)



SED= +/- 0.0058
d.f.= 116

Table 3.9. Concentration of surfactant (%), required to effect wetting of leaves of different brassicas in relation to temperature (average of different light intensities and leaf positions).

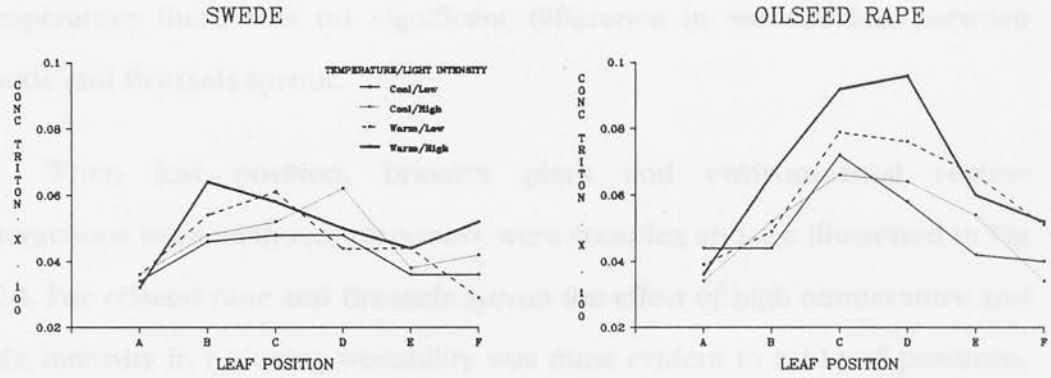
TEMPERATURE	LIGHT INTENSITY Low (4000lux)	High (8000lux)	Mean
Low (12°C)	0.046	0.047	0.046
High (18°C)	0.053	0.060	0.056
Mean	0.050	0.054	0.051

SED: Temperature +/- 0.0014 (d.f. = 4)
Light intensity +/- 0.0009 (d.f. = 7)
Temperature x Light intensity (at same level of temperature) +/- 0.0016 (d.f. = 7)
+/- 0.0013

Table 3.10. Concentration of surfactant (%), required to effect wetting of leaves in relation to temperature and light intensity during growth (average of three brassica types and leaf position).

TEMPERATURE	BRASSICA TYPE Swede	Oilseed rape	Brussels sprout	Mean
Low (12°C)	0.044	0.050	0.046	0.047
High (18°C)	0.047	0.064	0.058	0.056
Mean	0.045	0.057	0.052	0.051

SED: Temperature +/- 0.0014 (d.f. = 4)
Brassica type +/- 0.0010 (d.f. = 30)
Temperature x Brassica type (at same level of temperature) +/- 0.0018 (d.f. = 30)
+/- 0.0014



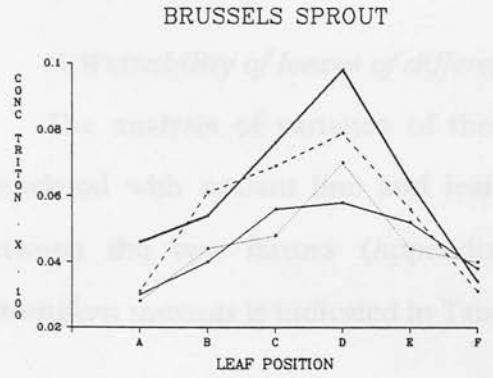


Fig. 3.18.

The concentration of surfactant required to effect wetting of leaves of different brassicas in relation to temperature, light intensity and leaf position

SED= +/- 0.005; d.f.= 224

In considering differences between brassicas, swede leaves on average were more readily wetted than Brussels sprout, oilseed rape leaves were most water repellant (Table 3.10). Temperature had an effect on all three brassicas, although differences were very slight on swede. While at the higher temperature differences between all three brassicas were evident, at the lower temperature there was no significant difference in wettabilities, between swede and Brussels sprout.

When leaf position, brassica plant and environmental regime interactions were analysed, responses were complex and are illustrated in Fig 3.18. For oilseed rape and Brussels sprout the effect of high temperature and light intensity in reducing wettability was most evident in mid-leaf positions. Differences in wettability on apical and basal leaves were less clear cut, tending to approach similar values for all environmental regimes. No consistent effect of environmental factors upon wettability was observed for swede. Leaves towards middle positions were most water repellant, but the effects of temperature and light intensity were very slight.

d. Wettability of leaves of different **gemmifera** *mutants.*

The analysis of variance of the data showed significant main effects associated with mutant line and leaf position but a significant interaction between the two factors (Appendix 3.4). The wettability of leaves of *gemmifera* mutants is indicated in Table 3.11.

Waxy phenotypes had a significantly reduced wettability in comparison with glossy phenotypes. Wettability values for the intermediate phenotypes were interposed between waxy and glossy estimates.

Water repellancy of mid-leaves, averaging over all mutants was

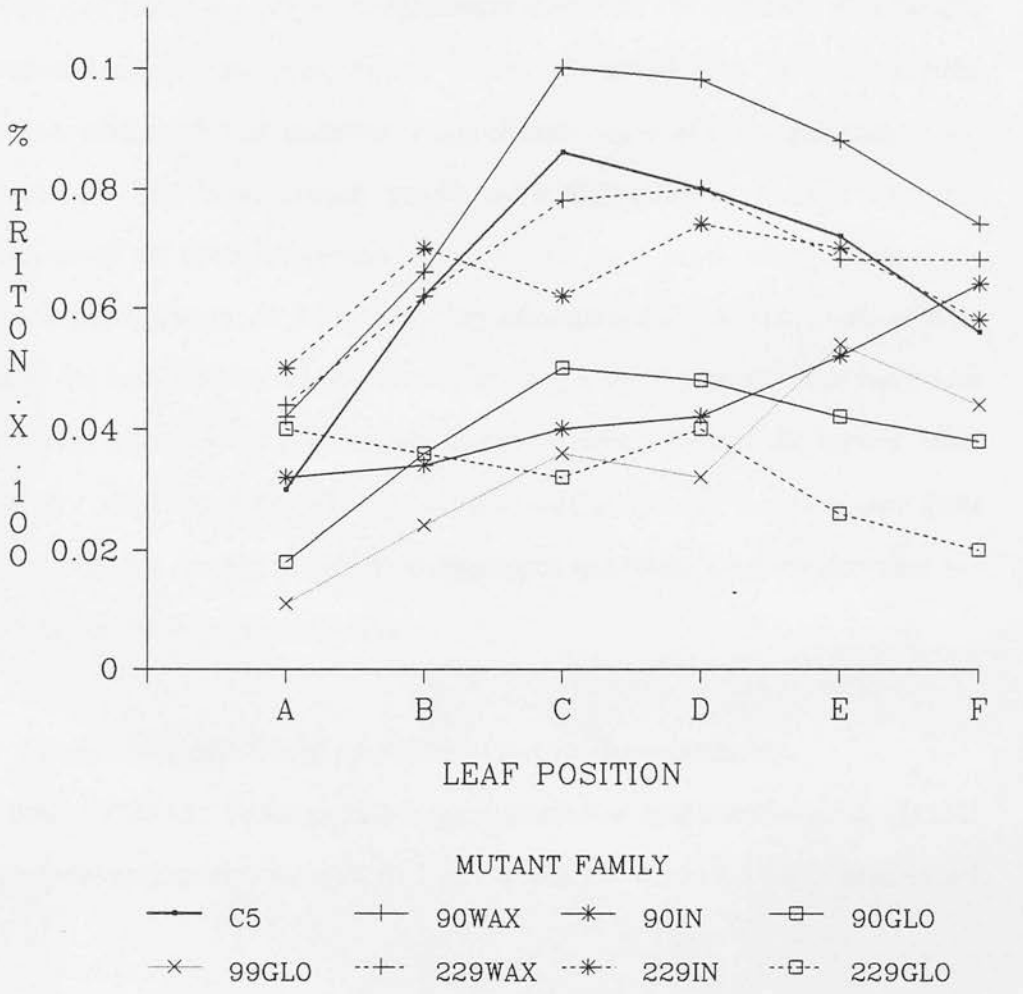
Table 3.11. Concentration of surfactant (%), required to effect wetting of leaves of gemmifera mutants (average of six leaf positions).

MUTANT LINE	CONC. OF TRITON- X -100 (%)
C5	0.064
90WAX	0.078
90INT	0.044
90GLO	0.039
99GLO	0.034
229WAX	0.067
229INT	0.064
229GLO	0.032
SED =	+/- 0.003
d.f. =	160

Table 3.12. Concentration of surfactant (%), required to effect wetting of leaves of gemmifera mutants in relation to leaf position (average of eight mutant lines).

LEAF POSITION	CONC. OF TRITON- X 100 (%)
A	0.033
B	0.049
C	0.060
D	0.062
E	0.059
F	0.053
SED:	+/- 0.0026
d.f=	28

Fig. 3.19. Concentration of surfactant (%) required to effect wetting of leaves from *gemmifera* mutants



SED= +/- 0.0074
d.f.= 160

significantly lower than those of apical and basal leaves (Table 3.12). Interactions between the two factors are illustrated in Fig 3.19. Over all leaf positions water repellancy was generally high on waxy phenotypes and low on glossy phenotypes. Relatively low wettabilities were usually most evident in mid-leaf positions, but especially for waxy phenotypes.

3.3.5. Permeability of leaf surfaces of swede, oilseed rape, Brussels sprout and **gemmifera** *mutants.*

a. Permeability in relation to brassica type and leaf position.

Conductivity assessments in relation to leaf position and brassica at 0, 10 and 24 hours post application are illustrated in Fig 3.20.

Only leaf position showed a significant effect for conductivity at 0 hours; mid leaves leaked less than apical or basal leaves. By 10 hours post application neither factor showed a significant main effect (Appendix 3.5). Nevertheless by the latter period trends were beginning to emerge whereby basal leaves of all three brassicas appeared to leak more electrolytes than apical or middle leaves. At 24 hours after application significant main effects were seen for leaf position. Conductivity levels on basal leaves of the three test brassicas was significantly higher than those on apical or middle leaves, with apical leaves of all three brassicas tending to exhibit greater permeability than middle leaves, but not significantly. Differences in conductivity of droplets on different brassicas were not significant.

b. Permeability of leaves of different **gemmifera** *mutants.*

Conductivity values for *gemmifera* mutants and leaf position at 0, 10 and 24 hours post application are detailed in Table 3.13, Table 3.14 and illustrated in Fig 3.21.

A significant level of variation in conductivity for mutant line but not leaf position was seen at the initial assessment (Appendix 3.6). Leaves of glossy phenotypes (90GLO, 99GLO, 229GLO) generally showed higher conductivities than waxy or intermediate phenotypes (Table 3.13).

Fig. 3.20. Conductivity of water droplets on leaf surfaces of brassicas in relation to leaf position

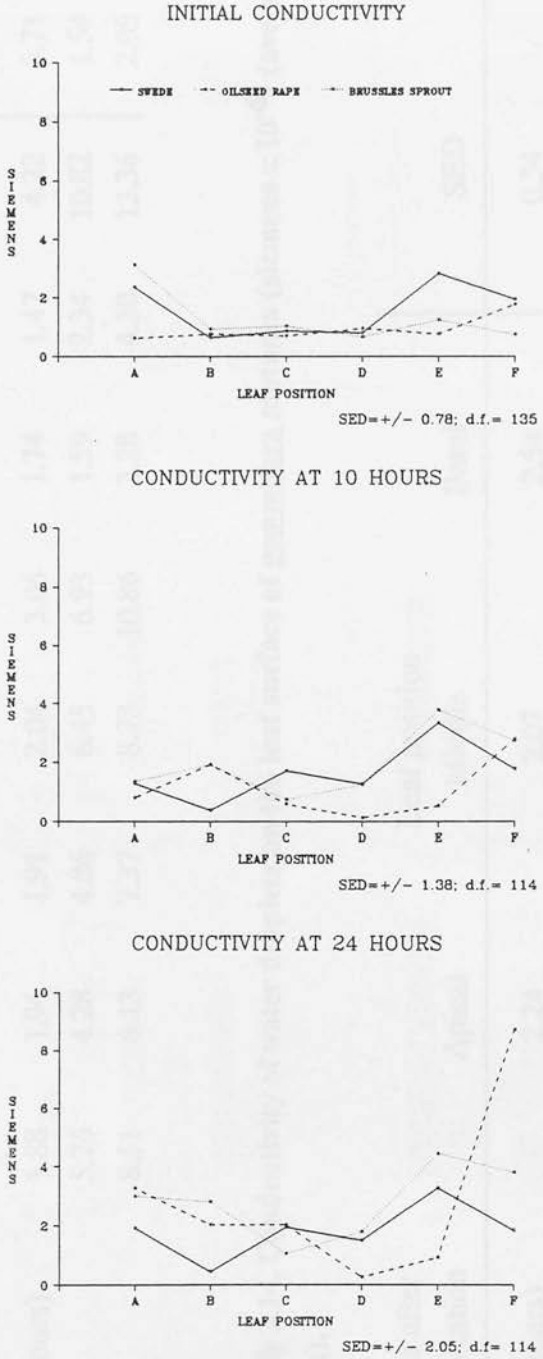


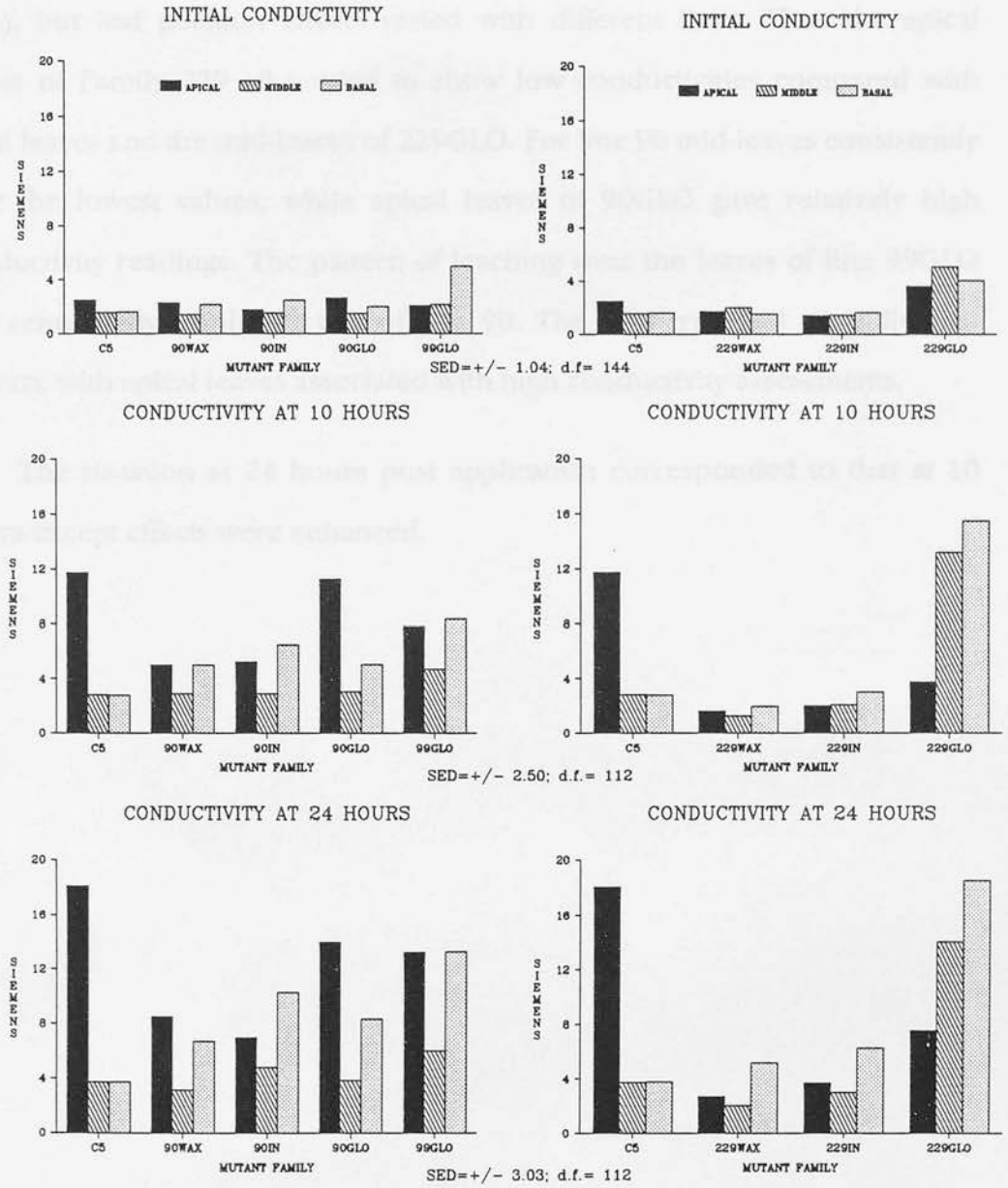
Table 3.13. Conductivity of water droplets on the leaf surface of gemmifera mutants (siemens x 10^{-6}) (average of three leaf positions).

Time after application	Mutant line C5	90W	90I	90G	99G	229W	229I	229G	SED	d.f.
0 (hours)	1.88	1.94	1.91	2.04	3.06	1.74	1.47	4.22	0.71	63
10	5.76	4.28	4.86	6.45	6.93	1.59	2.34	10.82	1.54	60
24	8.51	6.13	7.37	8.73	10.86	3.28	4.30	13.36	2.05	60

Table 3.14. Conductivity of water droplets on the leaf surface of gemmifera mutants (siemens x 10^{-6}) (average of eight mutant lines).

Time after application	Leaf position Apical	Middle	Basal	SED	d.f.
0 (hours)	2.24	2.07	2.54	0.34	144
10	6.04	4.10	6.00	0.85	112
24	9.33	5.08	9.04	0.97	112

Fig. 3.21. Conductivity of water droplets on leaves of *gemmifera* mutants



By 10 hours significant main effects and a significant interaction between mutant line and leaf position occurred (Appendix 3.6). Leaves from glossy mutants continued to show higher permeabilities than waxy mutants (Table 3.13), particularly the glossy phenotype of line 229. Middle leaves overall gave rise to lower conductivity values than either apical or basal leaves (Table 3.14), but leaf position effects varied with different lines. Thus the apical leaves of Family 229 all tended to show low conductivities compared with basal leaves and the mid-leaves of 229GLO. For line 90 mid-leaves consistently gave the lowest values, while apical leaves of 90GLO gave relatively high conductivity readings. The pattern of leaching over the leaves of line 99GLO was remarkably similar to that of line 90. The wild type had an individual pattern, with apical leaves associated with high conductivity assessments.

The situation at 24 hours post application corresponded to that at 10 hours except effects were enhanced.

3.3.6. Bioassay of the fungitoxicity of epicuticular wax fractions.

Growth of *Cladosporium* sp. on the P.L.C. plate was uniform and sporulation was profuse, except in one zone (Fig. 3.22). The area where growth was inhibited was consistent for all leaf positions of the three brassicas. R.F.s of this zone were calculated to be 0.74, and this corresponded with the ketone fraction of the waxes (Fig 3.7).

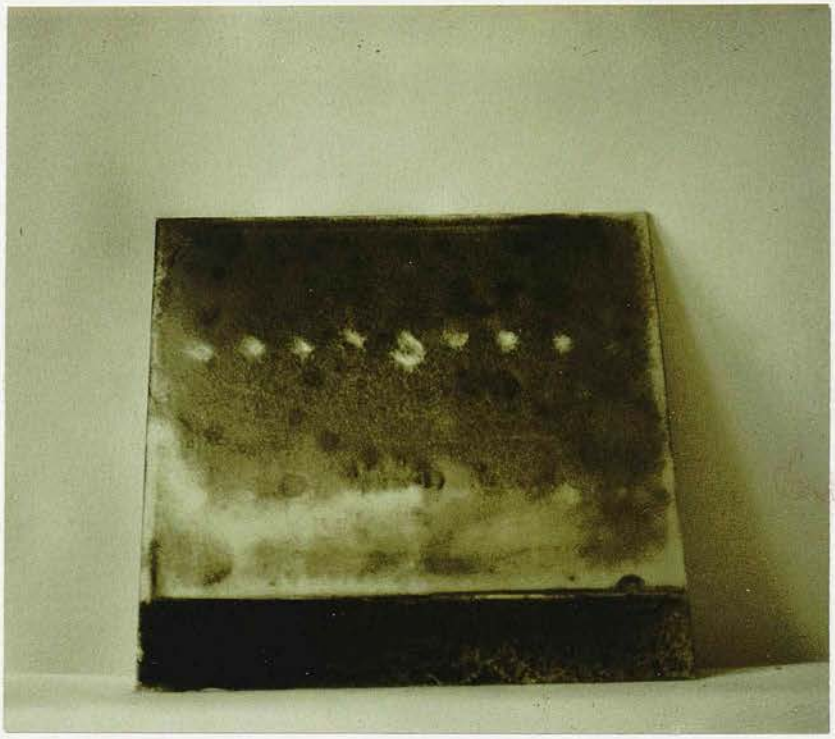


Fig. 3.18. Bioassay plate of brassica waxes inoculated with *Cladosporium* sp.

3.4. Discussion

The cuticle holds the primary interactive position between a plant and its aerial environment. Important functions assigned to the cuticle are the conservation of water, prevention of loss of metabolites by the phenomenon of leaching and a degree of protection against physical damage. Distribution and retention of substances, such as water and agrochemicals, at the leaf surface and their absorption into the plant are also greatly influenced. In addition the cuticle provides the first barrier to attack by potential pathogens. Manipulation of leaf surface characteristics, for the purposes of improving resistance levels of plants to foliar infection, requires an understanding of physical/ biochemical and physiological features. This series of experiments has concentrated mainly on the epicuticular wax region of brassica cuticles, appraising character and properties.

Wax development is a topic which has received detailed attention from many workers (Martin & Juniper, 1970s; Tulloch, 1973; Baker, 1974; Baker, *et al.*, 1975). The developmental process is not uniform and varies even within a species, but it is agreed that assimilation and exudation of a pre-determined "quota" of wax precursors occurs at an early stage and continues during leaf expansion (Juniper & Jeffree, 1983).

Results of the survey on wax quantities revealed this to be the situation with oilseed rape, Brussels sprout and swede, although maximum exudation of precursors occurred shortly after leaf emergence in the latter. Substantial volumes of wax precursors, mainly palmitate (Macey, 1970), are thought to be exuded during early growth of leaves owing to the cuticle being thin and fairly porous (Martin & Juniper, 1970). Exudation diminishes as the leaf expands

due to the thickening and hardening of the cuticular layer, thereby retarding extrusion (Freeman *et al.*, 1979). At leaf maturation secretion of precursors is believed to be complete, and thus ceases. Deposits decline rapidly after this stage presumably due to the removal of waxes by weathering and friction (Wilson, 1984; Baker & Hunt, 1985).

Quantities of wax per unit area on all three brassicas appeared to be negatively correlated with leaf area. Sharpest declines of wax took place from apical to mid-leaf positions which was during the period of greatest expansion, suggesting that secretion of wax precursors is unable to maintain rates equivalent to leaf expansion. The sudden increase in leaf area in basal leaves of Brussels sprout is probably due to the growth conditions under which the plants were grown and which are thought not to occur under field conditions.

The chemical composition of waxes is reported to play a major role in determining wax fine structure on the leaf surfaces (Juniper & Jeffree, 1983). In agreement with previous work (Macey, 1970; Baker, 1974; Jeffree *et al.*, 1976; Holloway *et al*, 1977) waxes from swede, oilseed rape and Brussels sprout were found to be of a complex nature, composed of eight analogous chemical groups, but in varying proportions. Crystalline structures on the three surfaces were much the same, except their densities varied. Use of a model system (Jeffree *et al.*, 1976) to crystallise plant waxes through a porous disc, showed that whilst some waxes crystallised in forms similar to that on the leaf surface, waxes of a more heterogeneous disposition required separate crystallisation of components before similar structures were observed. Based on their observations, the rodlets on leaves of the three brassicas were correlated with high quantities of secondary alcohols and

ketones. Aldehydes and primary alcohols were associated with the plate-like structures seen on leaves of swede. Despite the apparent lack of any plate configuration on leaves of oilseed rape and Brussels sprout, they were assumed to be present, but were probably obscured by the high density of rodlet waxes.

The possibility that hydrocarbons influence crystallinity at the surface cannot be ruled out. High amounts of hydrocarbons on the apical and middle leaves of swede, oilseed rape and Brussels sprout was coincidental with a high degree of crystallinity, seen on Scanning Electron Micrographs. Basal leaves, with relatively low quantities, were almost devoid of crystal structure, this being especially apparent for Brussels sprout. Although the relationship between crystal morphology and hydrocarbon content has been implied in the literature (for example Jeffree *et al.,* 1976), Hunt *et al.*, (1976) reject the idea, suggesting that hydrocarbons contribute primarily to the underlying wax film. Presumably then, loss of crystals would be due to such factors as weathering. In this respect, quantities of hydrocarbons would remain constant, whilst secondary alcohols and ketone would decrease. However, secondary alcohols increased with increasing leaf age and ketone counts remained fairly constant. It seems plausible to suggest that the loss of crystalline structures on basal leaves is not only due to effects of weathering but is also due to a change in wax chemistry. In addition, the findings from the present work confirm the association between hydrocarbons and rodlet conformations reported by other workers.

Waxes of the three brassicas were sensitive to environmental modification. Variation in size and density of crystals with increase in light intensity, complied with those previously found by Baker (1974). The

appearance of dendrites on surfaces of plants grown under high temperatures has also been reported by Whitecross & Armstrong (1972) for field rape, Baker (1974) for Brussels sprout, and Hunt *et al.* (1976) for *Clarkia elegans*, which has a similar wax chemistry and morphology as Brussels sprout.

Reactions to light intensity can perhaps be explained by its stimulatory effect on wax production. Macey (1970) found light prompted increases in the synthesis of palmitate, the primary wax precursor. Increases in size and density may be due to increases in volumes of wax at the surface. The modification of wax morphology by temperature is thought to be an expression of crystallisation rates. Slow crystallisation rates with low temperatures result in formation of linear conformations. Under high temperatures rates increase and favour structures resembling dendrites (Baker, 1974).

Jeffree *et al.* (1976) commented on the susceptibility of multicomponent waxes to environmental alteration and their failure to recrystallise on ceramic surfaces in the forms found on intact cuticles. They surmised that individual components most likely crystallised at different sites on leaf surfaces, which explained composite arrangements. This is of particular interest to present studies due to the implication that exudation rates may also be important in determining wax morphology. The mechanism by which precursors are delivered to the surface has yet to be resolved. Wax is perhaps carried through the cuticle in a solvent (Jeffree *et al.*, 1976) or alternatively enveloped in lipo- or glyco- transport proteins (Hallam, 1982). The concentration of each fraction at a particular site on the cuticle would therefore be determined by the rate of exudation and/or its solubility in the

carrier solvent or protein. On arrival at the surface the prevailing environmental conditions would influence crystallisation. Thus a variety of interactive forces are responsible for the variations in brassica wax crystal morphology.

Analysis of genetic control of wax composition and morphology highlighted the relationship. Loss of crystallinity in glossy phenotypes of mutant lines 90 and 229 correlated with reductions of ketones, secondary alcohols and hydrocarbons, and major increases in the aldehyde fraction. Similarly, glossy mutant 99 was characterised by reduced quantities of these fractions but high primary alcohols

Previous work on brassica wax mutants (Macey, 1970; Baker, 1974; Holloway *et al.*, 1977) disclosed two general morphological classes. Similar crystalline structures but at a much lower density are common to the *Rigo* field rape mutant (Holloway *et al.*, 1977) and the gl_2 mutant of *Brassica oleracea* (Baker, 1974). Subglaucous line gl_4 and gl_6 of *Brassica oleracea* (Baker & Holloway, 1975) and the *Nilla* mutant of field rape (Holloway *et al.*, 1977) have a series of wax plates raised from the surface.

Wax biosynthesis is based on an elongation-decarboxylation hypothesis, where two separate systems exist (Netting, Macey & Barber, 1972). In the first pathway long chain acids are precursors of ketones and hydrocarbons *via* conversion into secondary alcohols. The second system preferentially elongates branched-chain acids and incorporates them into primary alcohols and subsequently esters. The long chain acids from system one could be derived from intermediates of system two. Thus a single mutation affecting control of any of these systems could have dramatic effects on glaucousness.

Holloway *et al.* (1977) assumed conversion of C_{30} fatty acids to the corresponding alkane (C_{29}) was impaired in the *Rigo* mutant, resulting in a diversion of C_{30} acyl groups to aldehydes. Similar conclusions were made previously by Macey & Barber (1970) regarding mutant gl_2 of *Brassica oleracea* and are deemed to explain the glossiness of 90 and 229 mutant lines. Both lines have Go^c in their genotype, indicating that the aberration is at this locus.

Increased chain length or a loss of specificity for *anteiso-* components is usually given as the reason for glossiness in remaining mutants described by Macey, 1970; Netting, Macey & Barber, 1972 and Holloway *et al.*, 1977. In the latter *br-* alkanes exist in quantity with reductions in primary alcohols and esters. Mutant line 99GLO did not conform to either class. The large quantities of primary alcohols and esters suggested a shift from system one in favour of system two. It follows the mutation must cause a dramatic reduction in the synthesis of the enzyme responsible for conversion of system two intermediates into system one fatty acids. Therefore it is proposed that a new class be added to existing classification schemes to accomodate mutants resembling line 99GLO.

Biosynthetic pathways of waxes may also derive products which have microbial activity. Numerous reports are documented of substances on the surface of leaves inhibiting fungi (Dix, 1974; Godfrey & Clements, 1978; Parberry & Blakeman, 1978; Dix, 1979). The majority of these substances probably originate from the epidermal cells and are not associated with the cuticle. Effects of such "leachates" will be discussed later. Nevertheless, there are accounts of compounds extracted or co-extracted with wax which are fungitoxic (Blakeman & Atkinson; 1981), for example the surface wax of

beetroot is inhibitory to the germination of *Botyrtis cinerea* spores (Blakeman & Szternjnberg, 1973). The present study demonstrated that the ketone fraction of swede, oilseed rape and Brussels sprout wax was inhibitory to *Cladosporium* sp. and that activity was not lost as leaves aged. Considering that most of the reported inhibitory substances involve phenols, tannins or organic acids, the fungitoxic nature of the ketone fraction is unusual. Perhaps it is not the ketones themselves which are active but some breakdown product resulting from fungal metabolism. This would have important implications to the microflora on plant surfaces, selecting for those organisms which do not possess the enzymic apparatus necessary for the conversion. It would be attractive to suppose such organisms would be non-pathogenic, thereby restricting the number of potentially hostile fungi contacting the plant.

By far the most significant role of the epicuticular wax is the contribution it makes, with the cuticle proper, to the physical properties of the leaf surface. Of these, the water repellancy is a notable factor in water-proofing the plant, and hence influencing its absorption of water and chemicals. Measurement of the contact angle of water droplets on leaves is the usual estimate of their ability to shed water. The method was found to be both expensive and time consuming. The technique of Silva-Fernandez (1965), based on varying the surface tension characteristics of water in surfactant, was preferred.

Wettability is thought to be a function of macro- and micro- corrugations on the leaf surface. It is wax morphology and not quantity which is crucial (Fogg, 1947; Troughton & Hall, 1967). Water repellancy is greatest when the wax has a crystalline structure in the form of projecting rods or tubes (Troughton & Hall, 1967). Hence despite copious volumes of wax on apical

leaves they were found to be readily wetted.

Examination of SEMs in the present study showed a high degree of crystallisation on apical leaves yet these leaves showed a relatively low water repellancy. It must be noted that for protruding crystals to be effective as water repellants, there must be a sufficient density to prevent contact between water droplets and the cuticle proper. A "bloom" on the surface of glaucous leaves indicates high densities of crystals, but absence of a "bloom" does necessarily mean a waxless surface. Sub-glaucousness can be due merely to a reduction in structures. Differences observed in the wettabilities of the three brassicas under study can be interpreted by the degree of crystallisation on the surface. The presence of a "bloom" on oilseed rape and Brussels sprout implied a high crystal density, and so low wettabilities. On the other hand swede leaves which had a reduced "bloom", were easily wetted, even though they were crystalline according to SEMs. Only the conformation of structures was reliably defined by freeze-fracture and not the extent to which crystals covered the surface.

Factors affecting crystallisation have already been discussed. One additional aspect worthy of mention is the overall morphology of the three brassicas. Development of Brussels sprout is such that apical leaves are almost enclosed within the more mature leaves. Apical leaves of swede and oilseed rape are slightly more exposed. Transition of precursors to mature wax crystals requires physical influences of the environment (Martin & Juniper, 1970). The delay in crystallisation of apical leaves of Brussels sprout caused by limited exposure could explain their low water repellancy compared with swede and oilseed rape.

High wettabilities of basal leaves resulted from possible erosion or

changes in wax chemistry. Since extrusion of waxes was complete, any crystals lost would not be replaced. Many examples exist demonstrating the effect of weathering on waxes. Wilson (1984) drew attention to the damage to wax fine structure by wind abrasion. Baker & Hunt (1981; 1985) and Haines, Jernstedt & Neufeld (1985) found the impact of simulated rain droplets was sufficient to reveal large areas of the cuticle.

The effects of environmental factors on wettability were concurrent with their influence on crystal morphology. Significant increases in water repellancy of leaves from high temperature plants were associated with the appearance of dendritic structures which effectively covered the cuticle proper and restricted contact or spreading of water droplets. Greater water repellancy with increase in light intensity probably disclosed an increase in crystal number, a feature difficult to ascertain from SEMs. The areas of exposed cuticle on low temperature plants probably retained water droplets and allowed them to spread easily, regardless of any increase in crystallites. Thus significant increases in water repellancy were not observed with high light intensity at low temperatures.

The effects of environment on crystal morphology and therefore wettability were particularly well demonstrated when comparing brassica type and leaf position. Brussels sprout and oilseed rape leaves were significantly more water repellant than swede and mid-leaves more so than apical or basal leaves. However significant environmental influences on wettability were observed only on those leaves which had substantial coverages of crystals i.e. mid-leaves of Brussels sprout and oilseed rape.

Wettability of leaves of *gemmifera* mutants confirmed the property was dependent on surface features. All waxy phenotypes, including the wild type

(C5), showed significantly higher water repellancy indices than glossy varieties, while showing the characteristic trends with regard to leaf position.

The three mutants of line 90 supported the view that density of crystals is important in dictating "bloom" and wettability. The surface of the waxy phenotype had a complete coverage of crystals, a distinctive "bloom" and low wettability. The glossy phenotype, whilst not exhibiting a "bloom", was nonetheless crystalline, even though these were sparse. Consequently wettability was found to be high.

Application of a commercial surfactant reduced the water repellancy of all brassicas, however the effects were most emphasised on the mid-leaves, where there were believed to be the largest densities of wax crystals. The surfactant Agral is a "spreader". Therefore it alters the surface tension of water droplets allowing a greater interface between surface and droplet (Hassall, 1982). Rawlinson, Muthyalu and Turner (1978) found that besides increasing contact angles the herbicide Dalapon caused modifications of the wax. Wax analyis and Scanning electron microscopy showed the herbicide effected a decrease in the total amount of wax present and transformed its crystalline structure.

On account of Agral being applied 2 hours before testing, then the effects on leaves must have persisted for a period. Either residues remained on the leaf surface which maintained spreading of droplets or Agral brought about modifications in the epicuticular waxes similar to Dalapon. SEM studies or quantitative analysis were not done, therefore this could not be ascertained.

The cuticle augments stomata in regulating water loss from plants. It is

believed that 10% of total water is transpired through the cuticle (Martin & Juniper, 1970). The exudation of nutrients from cellular tissue to the surface by leaching is governed by the physical properties of the cuticle. Leaching is defined as "the removal of substances from plants by the action of aqueous solutions, such as rain, dew, mist and fog" (Tukey, 1971) and will be reviewed in more detail in a later chapter.

The thickness of the cuticle bears no relationship with the level of permeability (Martin & Juniper, 1970). Instead the degree of impregnation of the cuticle with wax is decisive (Jeffree, 1986). Transpiration loss is the usual criterion when estimating permeability (Martin & Juniper, 1970). For the proposes of this study it was thought to be more appropriate to measure directly changes in conductivity of water droplets on leaf disks as an index of solute leakage. This perhaps did not give an indication of water loss, but an impression of one microclimate factor which might influence the development of micro-organisms on the leaf surface.

Conductivity values for the three brassicas revealed even the waxiest leaves did not prevent exudation of electrolytes. The observation that basal leaves were significantly more permeable agrees with previous reports. Many reports have established the higher transpiration rate of older leaves despite their thicker cuticles. (Martin & Juniper, 1970; Hunt & Baker, 1982).

Degradation and damage to the epicuticular waxes and probably the cuticle itself occurred on basal leaves (c.f. SEMs). When wax was removed by abrasion (Denna, 1970; Wilson, 1984; Haines, Jernstedt & Neufeld, 1985) the transpiration rates of leaves under study significantly increased. Increases in transpiration of these leaves and in conductivity of droplets on leaves of the three brassicas could occur from removal of hydrophobic materials in wax;

ketones and hydrocarbons are ascribed as being efficient water barriers.

The tendency for apical leaves to be more permeable is slightly more confusing, considering they have maximum deposits, in proportions favouring water retention. It seems diffusion of water through the cuticle is complicated by crystalline forms on the superficial wax. The series of experiments by Chamber & Possingham (1963) showed epicuticular waxes on grapes existed as overlapping plates. They proposed that water diffused in the liquid phase through the parenchyma, pectin and cuticle layers until it reached the platelet region. From here, they suggested that it vaporised; droplets or films could be due to the hydrophobic nature of the wax. Hence the diffusive continuum was broken delaying transfer to the surface. Similar theories could be applied to permeability of apical leaves, on account of areas bereft of crystals. Alternatively immature cuticles, with little cuticular wax may be the explanation. Overall the experiment did not signify whether epicuticular or cuticular wax was most important in determining permeability. If the epicuticular wax exerts the greater control then matching trends as those obtained for wettability would be expected.

Conductivity values for *gemmifera* mutants resembled their wettability patterns implying like forces were responsible. Assessments were significant for mutant lines (and the apical leaves of brassica cultivars) even at 0 hours. The leaves were not washed before deposition of droplets. Any electrolytes already on the surface, not unexpected since leaching is a continuous, physical process, would ionise in the droplet and contribute to the readings. In future studies therefore washed leaves should be used at 0 hours.

At 10 hours leaves from all glossy mutants were more permeable than waxy lines. Thus a negative correlation between glaucousness and

permeability could be drawn. On average mid-leaves were more permeable than apical or basal leaves. Whether the differences were significant depended on mutant line. Apical leaves from the wild type, lines 90 and 99GLO showed higher or comparable values as basal leaves. The opposite was true for line 229. The developmental morphology was once more thought to be the reason. Morphology of line 90 and 99GLO is akin to the wild type, whilst that of 229 was more like swede *i.e.* the apical leaves were not protected by older leaves.

Recordings at 24 hours emphasised those at 10 hours. The differences in wax structure which accounted for changes in wettability probably applied to permeability. Meanwhile it is still appreciated that the cuticular wax also exercises considerable control over the processes of cuticular transpiration and leaching.

Understanding chemical and physical properties of the leaf surface wax has demonstrated the layer is not a static structure, but dynamic, undergoing continuous change through various internal and external factors. Practical implications thus are many. For example using wax structure as a criterion in taxonomy is not advised. Waxes can alter structure through environmental forces or can lost simply by the action of rain.

Amount and distribution of chemicals deposited on the surface are governed by wetting properties of the surface. Wetting agents may modify surface waxes, and in turn surface properties, either by emulsification/solubilisation, leading to pathways through which polar molecules enter symplastic tissue, or by physical removal by impaction of spray droplets. The microclimate, created in part by the epicuticular wax, directs growth of surface epiphytes and potential pathogens up to the stage of

penetration. How pathogens react to different surfaces, in addition to mechanically transformed and naturally transformed surfaces, will now be explored in the following chapters.

CHAPTER 4

INFECTION BY TWO ALTERNARIA SPECIES AND ERYSIPHE CRUCIFERARUM IN RELATION TO BRASSICA LEAF SURFACE CHARACTERISTICS

4. INFECTION BY TWO ALTERNARIA SPECIES AND ERYSIPHE CRUCIFERARUM IN RELATION TO BRASSICA LEAF SURFACE CHARACTERISTICS

4.1. Introduction

The phylloplane represents the initial site of contact between a foliar pathogen and a potential host plant. In the habitat adjacent to the leaf surface, the phyllosphere, the early development of a pathogen is subject to the influence of physical, chemical and biological factors, which in turn are governed by the plant, its environment and their interaction. While having significance throughout the life cycles of a pathogen and its host, conditions during the early establishment phase of infection are obviously crucial in determining the eventual levels of disease incidence and severity which might occur.

The leaf surface plays host to a heterogeneous population of micro-organisms, which exhibit a broad spectrum of relationships with the plant and each other. Many of these are true commensals and assume an exclusively saprophytic lifestyle. Others have the ability to parasitise plant tissues, and may inflict varying degrees of physical and physiological damage on their hosts. Microbial interactions within the phylloplane may influence the behaviour of disease causing organisms (Fokkema, 1976; 1981; Skidmore, 1976). Pace & Campbell, (1974) found that the saprophytic fungi *Aureobasidium pullulans* and *Epicoccum nigrum*, leaf surface epiphytes of cabbage, were antagonistic to *Alternaria brassicicola*. When inoculated onto

leaves they reduced infection by the pathogen. However, this aspect of the phyllosphere is not considered further in the present study.

A simple classification for epiphytic microbes found on leaf surfaces was devised by Leben (1965) who distinguished "resident" organisms which are normally present from "casual" organisms which are deposited passively by external forces. Most of the phylloplane microflora are of the casual kind and it is into this category that phytopathogenic fungi are placed. Disease-causing fungi, however, require a series of critical conditions to realise their pathogenicity potential. Such conditions relating to the environment and to the host will in the first instance affect events at the surface of the plant.

Among the environmental factors which may exert an important effect on fungal development are temperature and humidity. Temperature will affect all stages of fungal development (Colhoun, 1973), including those processes involved in the establishment of infection. Different fungi, or even different races within the same species, may have different temperature optima for each developmental phase. Although different stages of growth of the same fungus may have different temperature requirements, the occurrence and severity of a disease can generally be linked to a particular temperature regime. Thus dark leaf spot of brassicas, caused by *Alternaria brassicicola*, is favoured by high temperatures and is more extensive in summer. Light leaf spot (*Pyrenopeziza brassicae*) of brassicas, on the other hand, is most destructive in Autumn when temperatures are cooler (Harthill & Cheah, 1984).

As with temperature, different fungi, or different events in the infection cycle of the same fungus may have different humidity requirements. Yarwood (1956) suggests that foliar pathogens can be classified into four main,

although not completely distinct, groups based on their requirements for moisture or high humidity. In the anthracnose group (acervular fungi with slimy spores) high humidity is required for sporulation, spore dispersal and invasion of the host plant; the downy mildew group require it for sporulation and invasion; in rust fungi it is required for invasion; the powdery mildews do not require high humidity for any of these processes. In some infections, interactions between temperature and moisture are found. For example, in the case of apple scab (*Venturia inaequalis*) the period of leaf wetness necessary for establishment of infection declines as temperature rises (Mills & La Plante, 1954).

Other factors which may affect the behaviour of fungal pathogens at leaf surfaces include light, the gaseous composition of the atmosphere and pH. Natural visible light appears to have little influence on spore germination (Tarr, 1972) but ultraviolet rays are harmful and light above 600 nm (red of the spectrum) can be inhibitory. Moreover, germ-tubes of some fungi are negatively phototropic. It seems that the spores of many fungi are tolerant of a wide range of pH, although the overall effects may be complicated by nutrient availability and other factors.

Foliar fungal pathogens may enter into leaves through wounds, natural openings (notably stomata) or by direct penetration through the cuticle. The importance of the cuticle as an obstacle to invasion by disease-causing organisms has been a subject of protracted debate. It is now generally accepted that, by its nature, the cuticle does not present a serious barrier to penetration, except when the structure is thickened and/or hardened (Martin, 1964). According to Tarr (1972),, the fact that more pathogenic fungi penetrate directly through the cuticle rather than through stomata substantiates this view.

Reports of different workers would indicate that the role of the cuticle as a mechanical barrier to invasion is significant in some plant-pathogen interactions but not in others. Varietal resistance of strawberry leaves to *Sphaerotheca macularis* was correlated by Peries (1962) with hardness of the cuticle: the cuticle of *Fragaria chiloensis*, a resistant species was reported by Jhooty & M^{C}Keen (1965) to be up to seven times thicker than *F. ovalis*, which was readily attacked. Blackspot disease (*Diplocarpon rosae*) of rose was found to be enhanced on the adaxial surface of leaves which had been treated with fine carborundum powder (Castledine, Grout and Roberts, 1981). The abrasions scored the cuticle but did not disrupt the underlying epidermal cell walls. Prasanna (1984) observed that when the outer wax layers were rubbed from half of the surface of oilseed rape leaves prior to spraying with *Alternaria brassicae*, infection developed more extensively on the wiped side of the leaf. In these examples it was inferred that the mechanical properties of the intact cuticle provided a barrier to infection. In contrast, penetration of rice plants by *Pyricularia oryzae* seems to bear no relation to thickness of cuticle (Veearagharon, 1983). Although the cuticle thickened with leaf age, no difference in the reaction of plants of resistant or susceptible cultivars followed.

Tarr (1972) has suggested that some cuticles may contain fungitoxic substances. Thus they might act as chemical as well as physical deterrents to pathogens. In support of this view he refers to reports of the toxicity of cutin acids of *Citrus* lime to *Gloeosporium limetticola* (wither tip), and of the association of cuticular waxes with the high resistance of *Ginkgo biloba* to disease.

The cuticle may often be of more significance in determining the success or failure of foliar pathogens through its indirect, conditioning effect on the

phylloplane environment. Due to its influence upon water repellancy, the cuticle and in particular the epicuticular wax layer will have an influence upon not only deposition (Heather, 1967; Maddock, Ingram and Gilligan, 1981) but also the availability of water for early fungal development. Furthermore, according to Blakeman (1973), the reduction in wettability and permeability of the cuticle caused by epicuticular waxes, in turn reduces the availability of leachates. Thus the epicuticular waxes may affect spore germination and further development by reducing diffusion of substances, which can be metabolised by both saprophytic and parasitic fungi, into water droplets on the leaf surface. Conn & Tewari (1989) proposed that the wax on canola (cultivars of *Brassica campestris* and *Brassica napus*) in the form of a fluffy layer may affect germination by impeding movement of foliar exudates to the surface.

Since the physical properties of the leaf surface are recognised to have possible direct or indirect influence on pathogen development, any alteration to surface structures would be likely to affect the infection process. Rawlinson *et al.* (1978) drew attention to the increased incidence of light leaf spot on oilseed rape which had been previously treated with the herbicide Dalapon. The combined effects of Dalapon on epicuticular waxes (see Chapter 3.4) increased the wettability of the leaf surface. It was proposed that the increased wettability resulted in retention of conidia on the leaf and other surfaces. More recently Munro (1984) noted that *Alternaria* leaf spot occurred more frequently in plots of cabbage which had been treated with the fungicide Tridemorph, compared with untreated plots. The effects were associated with the wetting of the surface by the surfactant used in the fungicide formulation, which favoured retention and development of the pathogen.

In the previous chapter some effects of environmental factors, as well as

host genotype, on the surface characteristics of brassica plants have been indicated. In this chapter these surface characteristics are considered further in relation to infection by three fungi, *Alternaria brassicicola, Alternaria alternata* and *Erysiphe cruciferarum.*

Alternaria brassicicola is the cause of dark leaf spot of cruciferous plants (Holliday, 1989), giving rise to dark brown to black, circular, zonate spots, usually 1 to 10 mm in diameter, on leaves (Ellis, 1971). It can affect all stages of growth, having injurious effects on seed, seedlings, stems, inflorescences and pods (Prasanna, 1984). It has a wide geographical distribution and is recognised as a major pathogen of seed crops of members of the Cruciferae, particularly cabbage and cauliflower, in temperate regions. The fungus produces dark brown conidia in profusion which are developed in chains. The spores taper slightly towards the apex or are obclavate in shape, with a small beak usually almost non-existent. The spores are multiseptate, with up to 11 cross walls and a few longitudinal ones, and measure up to 130 μm in length. The fungus is classified in the family Dematiaceae of the Hyphomycetes.

The pathogen is primarily seed-borne but can persist in soil debris and is dispersed by air-borne spores. Humpherson-Jones & Hocart (1983) indicated an optimum temperature of 25 ^{o}C for infection and the need for free water for disease development. From spore germination on leaves, cuticular penetration is most frequent and is preceded by formation of appressoria (Dixon, 1981). Some stomatal penetration may occur. Following invasion of the epidermal layer, hyphae ramify between and within cells of the mesophyll and palisade tissue until the entire leaf is parasitised. At an early stage of infection epidermal cells become necrotic and the underlying parenchyma tissue collapses ahead of the advancing hyphae. The fungus does

not produce haustoria. Production of pectase and celluase enzymes by the pathogen has been demonstrated (Shohet, 1985) and phytotoxins have been implicated in its pathogenicity (Hodgkin & MacDonald, 1986).

Alternaria alternata is described by Ellis (1971) as an extremely common saprophyte found on many kinds of plants as well as substrata. It may, however, also have pathogenic attributes probably most associated with host damage (Holliday, 1989). Parry (1990) described it as weakly pathogenic on sugar beet, causing target-spotting and browning of the margins of the leaf. It is one of the fungi which can cause black point of wheat (Holliday, 1989), where the end of the grain is shrivelled and dark coloured. The conidia of this fungus are again formed in long chains and are pale brown in colour, generally ellipsoid with a short beak. The spores, which are up to 63 μm long, have up to eight transverse septa, and usually several longitudinal or oblique cross walls.

Erysiphe cruciferarum, in contrast to the *Alternaria* species, is a strict biotroph and cannot be grown axenically. It is a member of the Erysiphaceae and like other powdery mildew fungi appears as a whitish, mealy cover over leaves and other aerial parts of cruciferous plants (Dixon, 1981). The cover comprises mycelium which gives off singly or in short chains hyaline, cylindrical or elliptical conidia up to 41 μm in length. The spores are air-borne, germinating to produce usually several germ-tubes, and resulting in a network of mycelium over the surface. Internal growth is confined to haustoria in the epidermal cells. Powdery mildew has a widespread distribution and can be severe at times, reducing yield and impairing the quality of such vegetable crops as Brussels sprout. A unique feature of the spores of powdery mildew fungi in general is their ability to germinate and infect in the absence of external liquid water (Butt, 1978). However, germ-

tubes may be more sensitive to moisture stress than their parent conidia. Dixon (1981) indicated a lack of data on epidemiological aspects of *E. cruciferarum*, but noted that traditionally epidemics are associated with low RH and water stress within the host. More recently Parry (1990) observed that mildew epidemics were most likely in early summer when the temperatures are between 17 and 20 ^{o}C and the relative humidity is high.

Four experimental studies were carried out which complemented studies described in the previous chapter on the effect of brassica type, leaf position, surfactant treatment, the temperature and light intensity conditions of plant growth and *gemmifera* mutant line on leaf surface characteristics. The broad aim of this experimental section was to attempt to relate these effects to results of disease assessment studies.

4.2. Materials and methods

Four experiments were carried out with each of the three pathogens, using similar inoculation and assessment procedures in each experiment. With *Alternaria brassicicola* and *Alternaria alternata* a 20 ml spore suspension, prepared as previously described in Chapter 2.4, was inoculated onto leaf disks using a Badger 500-E-X Airbrush. Petri-dishes containing disks were placed in moist chambers and incubated in a Fisons growth cabinet set at 20 ± 2 ^{o}C with a 14 hour daylength. After two days disks were visually examined for disease and assessed by one of two techniques:

i. Counts of lesions on the leaf surface.

A Kyowa stereoscan microscope, fitted with a graticule eyepiece grid was used to magnify (x 40) infected leaves (Fig 4.1). Each individual lesion was counted, such that total lesion number represented an estimate of disease severity (Experiment 4.2.1 and 4.2.2).

ii. Disease assessment scale.

A disease assessment scale was developed, based on the method of Prasanna (1984). The range of estimates was from 0-10, where 0 corresponded to no visible symptoms and 10 corresponded to three-quarters or more leaf disk infected (Fig. 4.2) (Experiments 4.2.2, 4.2.3 and 4.2.4).

Conidia of *Erysiphe cruciferarum* were inoculated onto leaf disks as previously described in Chapter 2.4. Petri-dishes containing disks were then placed directly into a Gallenkamp incubator set at 18 ^{o}C with a 12 hour daylength. After an incubation period of 5 days disks were assessed visually. The degree of powdery mildew infection was estimated according to the

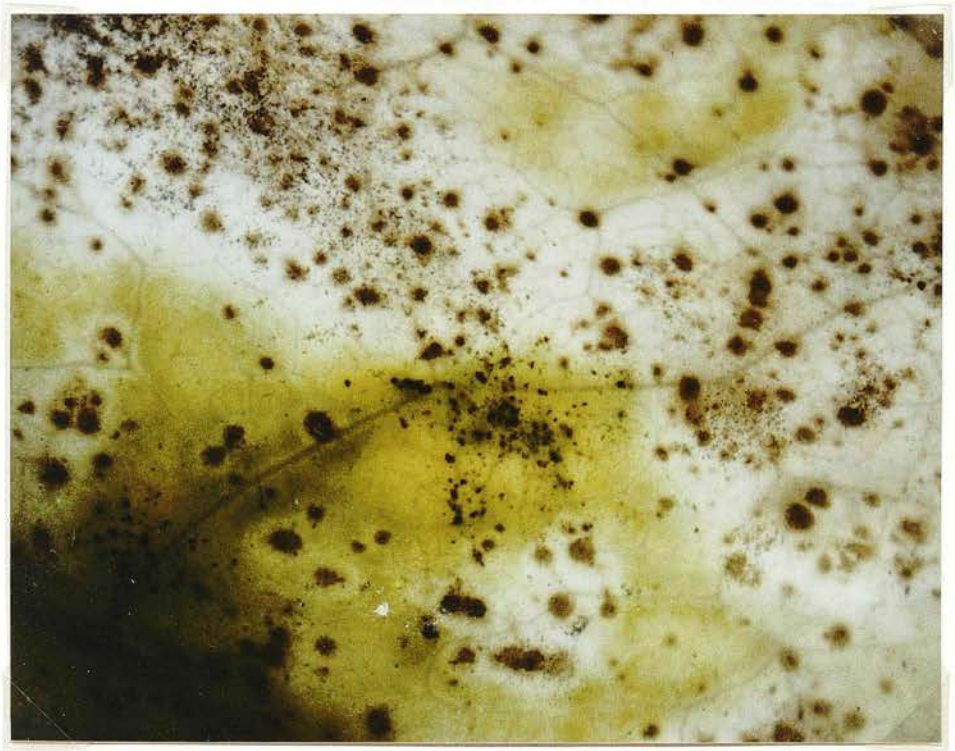


Fig. 4.1. Lesions of *Alternaria brassicicola* on mid-leaf disk of swede (x 40 magnification).

Fig.4.2. Alternaria disease assessment scale (0-10) for leaf disk inoculation studies.

0 = No visible symptoms

1 = Very occasional small specks

2 = Scattered small specks

3 = Specks increasing in size and number

4 = Specks or spots tending to merge

5 = More general merging of spots

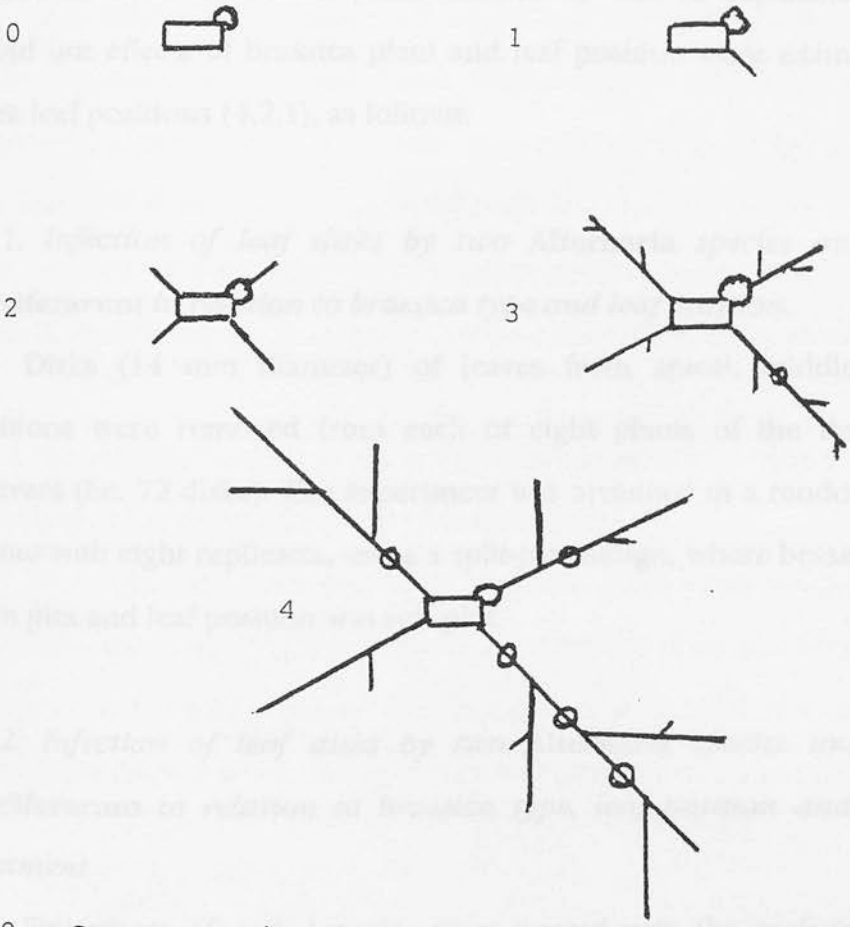
6 = Merged spots affecting about one-quarter of area

7 = Merged spots affecting about one-third of area

8 = Distinct large spot with yellow halo over one-half of area

9 = About three-quarters of area affected

10 = Almost all area affected



0 = Spore germination, no fungal growth beyond appressorium formation
1 = Restricted mycelial growth, no sporulation
2 = Limited mycelial growth, no sporulation
3 = Moderate mycelial growth, no sporulation
4 = Abundant mycelium, moderate sporulation
5 = Abundant mycelium, abundant sporulation

Fig.4.3. Diagrammatic representation of the 0-5 disease assessment scale for Erysiphe cruciferarum

method of Munro (1985) using the key illustrated in Fig 4.3.

The four experimental studies in which each of the pathogens were examined, used equivalent plant material to that of experiment 3.2.4a to 3.2.4d but effects of brassica plant and leaf position were examined at only three leaf positions (4.2.1), as follows:

4.2.1. Infection of leaf disks by two **Alternaria** *species and* **Erysiphe cruciferarum** *in relation to brassica type and leaf position.*

Disks (14 mm diameter) of leaves from apical, middle and basal positions were removed from each of eight plants of the three brassica cultivars (i.e. 72 disks). The experiment was arranged in a randomised block lay-out with eight replicates, using a split-plot design, where brassica type was main plot and leaf position was sub-plot.

4.2.2. Infection of leaf disks by two **Alternaria** *species and* **Erysiphe cruciferarum** *in relation to brassica type, leaf position and surfactant treatment.*

Five plants of each brassica were treated with the surfactant Agral as described in chapter 3.2.4b. Disks (14 mm diameter) of leaves from positions assigned A-F as before were removed from each of five treated brassica plants and five untreated brassica plants, the latter being designated as controls. Control and treated leaf disks from the different leaf positions of the three brassicas were placed in a randomised block arrangment of five replicate Petri dishes. The design was a split-split plot design with treatment as main plot, brassica plant as sub-plot and leaf position as sub-sub-plot.

4.2.3. Infection of leaf disks by two **Alternaria** *species and* **Erysiphe cruciferarum** *in relation to brassica type, leaf position and environmental factors.*

Leaf disks from positions A-F were taken from each of five plants of the three brassicas which had been raised under different environmental conditions (Chapter 2.2). For each temperature/light intensity combination one leaf disk from each position of each brassica was placed at random in each of five blocks with a split-split-split-plot design with environment treatment as main plot, brassica as sub-plot and leaf position as sub-sub-plot.

4.2.4. Infection of leaf disks from different **gemmifera** *mutant lines by two* **Alternaria** *species and* **Erysiphe cruciferarum.**

Disks (14 mm diameter) were removed from positions A-F of each of five plant replicates of different *gemmifera* mutant lines and of the wild type. These were randomly placed in Petri dishes in a split plot design where *gemmifera* mutant was a main plot and leaf position was a sub-plot.

4.3. Results

4.3.1. Infection of leaf disks by two **Alternaria** *species and* **Erysiphe cruciferarum** *in relation to brassica type and leaf position.*

Alternaria brassicicola.

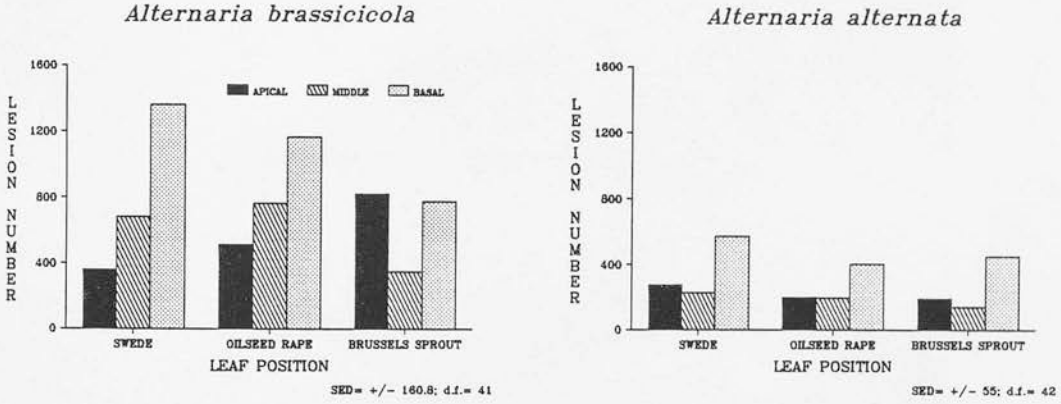
Analysis of the data for the effect of brassica and leaf position showed a significant difference in lesion numbers associated with leaf position, and a significant interaction between leaf position and host type (Appendix 4.1). Basal leaves gave more infection than middle leaves in all three brassicas (Fig. 4.4). Apical leaves of Brussels sprout also gave relatively high infection rates, whereas those of swede and oilseed rape gave the lowest disease scores in comparison with other leaves of the same plant. Swede and oilseed rape showed more infection than Brussels sprout on basal and middle leaves but the converse applied for apical leaves (Figs 4.5, 4.6 and 4.7).

Alternaria alternata.

In general fewer lesions were produced from *A. alternata* inoculations in comparison with *A. brassicicola* (Fig. 4.4). Lesion number in this case varied with brassica plant and leaf position (Appendix 4.1). Swede showed more infection than oilseed rape and Brussels sprout, and basal leaves showed greater numbers of lesions compared with other leaf positions.

Erysiphe cruciferarum.

From the analysis of the data for powdery mildew infection, infection levels were found to vary significantly with both brassica type and leaf position but there was a significant interaction between the two factors



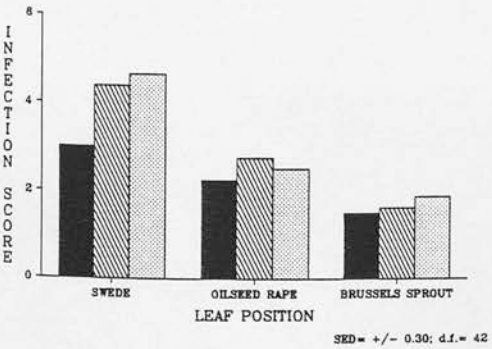


Fig. 4.4.

Infection rates of *Alternaria brassicicola*, *Alternaria alternata* and *Erysiphe cruciferarum* on leaves from different brassicas in relation to leaf position.

Figs 4.5 - 4.7. Leaf disks infected with *Alternaria brassicicola*. Top row- apical leaves, middle row- mid-leaves, bottom row- basal leaves.

Fig. 4.5. Swede.

Fig. 4.6. Oilseed rape.

Fig. 4.7. Brussels sprout.

Fig. 4.5.

Fig. 4.6.

Fig. 4.7.

Figs 4.8 - 4.10. Leaf disks infected with *Erysiphe cruciferarum* (x 40 magnification).

Fig. 4.8. Swede.

Fig. 4.9. Oilseed rape.

Fig. 4.10. Brussels sprout.

Fig. 4.8.

Fig. 4.9.

Fig. 4.10.

(Appendix 4.1). At all leaf positions swede had a higher disease score than oilseed rape which, in turn, showed more infection than Brussels sprout (Figs 4.4, 4.8, 4.9 and 4.10). With respect to leaf position effects, middle or basal leaf infection levels were on average higher than those on apical leaves. However the effect of leaf position was significant only for swede.

4.3.2. Infection of leaf disks by two **Alternaria** *species and* **Erysiphe cruciferarum** *in relation to brassica type, leaf position and surfactant treatment.*

a. Alternaria brassicicola.

Analysis of variance of the data for lesion numbers showed significant main effects of surfactant treatment and leaf position. There was, however, a significant interaction between these two factors and between leaf position and brassica type (Appendix 4.2). Overall, surfactant treatment increased lesion numbers and basal leaves gave rise to more lesions than apical leaves (Table 4.1). However, the effect of surfactant treatment was significant only on middle leaves; treatment tended to reduce the differences associated with leaf position.

As with previous observations (Chapter 4.3.1), a significant interaction was found between brassica plant and leaf position (Fig 4.11). Lesions on swede and oilseed rape increased in number as leaves aged, from sub-apical towards basal positions. On the other hand leaves of Brussels sprout showed relatively little difference in their reaction to inoculation although, compared with other leaves, higher numbers of lesions were found at the extreme basal and sub-apical positions. Of the three brassicas, fewer lesions tended to be produced on basal leaves of Brussels sprout than on equivalent leaves of the other brassicas. Nevertheless Brussels sprout exhibited more infection on sub-apical leaves.

Alternaria alternata.

Significant main effects were seen for surfactant treatment and leaf

Table 4.1. Number of lesions produced by Alternaria brassicicola in relation to surfactant treatment and leaf position (average of three brassicas).

SURFACTANT TREATMENT	LEAF POSITION A (Apical)	B	C	D	E	F	Mean
Untreated	642	620	497	769	986	1358	812
Treated	912	847	1158	1444	1200	1254	1136
Mean	777	733	827	1106	1093	1306	974

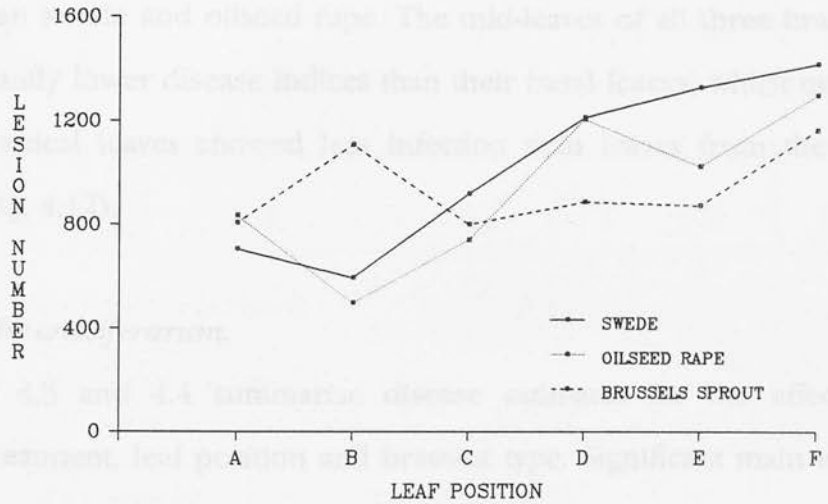
SED:	Surfactant treatment	+/- 87.9	(d.f. = 4)
	Leaf position	+/- 99.2	(d.f. = 114)
	Surfactant treatment x Leaf position	+/- 155.4	(d.f. = 114)
	(at same level of surfactant)	+/- 140.3	

Table 4.2. Infection scores produced by Alternaria alternata in relation to surfactant treatment and leaf position (average of three brassicas).

SURFACTANT TREATMENT	LEAF POSITION A (Apical)	B	C	D	E	F	Mean
Untreated	2.8	1.6	1.5	3.1	4.4	5.7	3.2
Treated	3.5	4.0	3.6	4.1	5.8	6.6	4.6
Mean	3.1	2.8	2.6	3.6	5.1	6.2	3.8

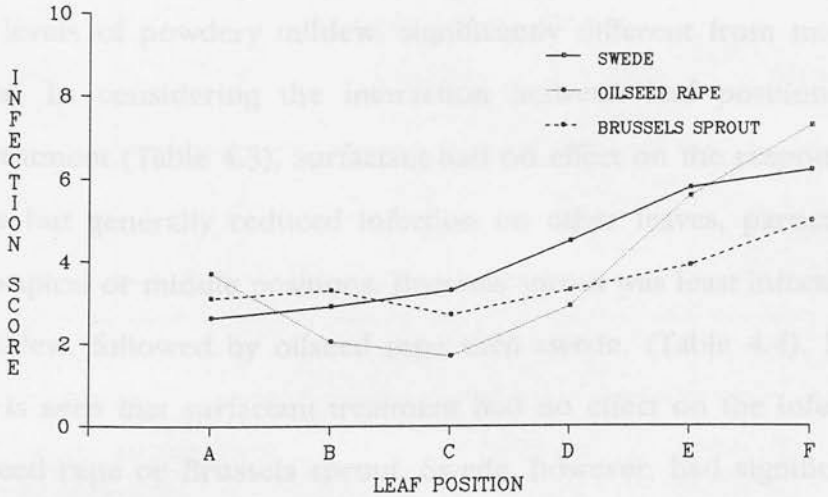
SED:	Surfactant treatment	+/- 0.24	(d.f. = 4)
	Leaf position	+/- 0.45	(d.f. = 116)
	Surfactant treatment x Leaf position	+/- 0.63	(d.f. = 116)
	(at same level of surfactant)	+/- 0.64	

Fig. 4.11. Numbers of lesions produced by *Alternaria brassicicola* in relation to brassica type and leaf position (averaged for surfactant treatment)



SED= +/- 176.4; d.f.= 114

Fig. 4.12. Infection scores of *Alternaria alternata* in relation to brassica type and leaf position (averaged for surfactant treatment)



SED= +/- 0.63; d.f.= 116

position (Appendix 4.2; Table 4.2). More disease, at a significant level, occurred on treated leaves compared with untreated and on basal leaves compared with apical or mid-leaves. Brassica type and leaf position showed a significant interaction (Fig. 4.12). Mid-leaves of oilseed rape gave lower scores than the other brassicas, while basal leaves of Brussels sprout had less infection than swede and oilseed rape. The mid-leaves of all three brassicas had significantly lower disease indices than their basal leaves, whilst only on swede did apical leaves showed less infection than leaves from the mid-positions (Fig. 4.12).

Erysiphe cruciferarum.

Tables 4.3 and 4.4 summarise disease estimates for the effects of surfactant treatment, leaf position and brassica type. Significant main effects are shown for all three factors but significant interactions occurred between them in most cases (Appendix 4.2). Surfactant treated leaves overall were less infected than untreated (Table 4.3). With respect to leaf position, an increase in disease incidence with older, basal leaves was obtained. Basal leaves had the highest levels of powdery mildew, significantly different from mid- or apical leaves. In considering the interaction between leaf position and surfactant treatment (Table 4.3), surfactant had no effect on the response of apical leaves but generally reduced infection on other leaves, particularly those in sub-apical or middle positions. Brussels sprout was least infected by powdery mildew, followed by oilseed rape then swede. (Table 4.4). From Table 4.4 it is seen that surfactant treatment had no effect on the infection level of oilseed rape or Brussels sprout. Swede, however, had significantly less disease on treated leaves, which gave no more infection than leaves of oilseed rape.

Table 4.3. Infection scores produced by Erysiphe cruciferarum in relation to surfactant treatment and leaf position (average of three brassicas).

SURFACTANT TREATMENT	LEAF POSITION A (Apical)	B	C	D	E	F	Mean
Untreated	1.0	1.7	1.6	1.9	2.1	2.2	1.7
Treated	1.0	0.3	0.7	1.1	1.2	1.7	1.0
Mean	1.0	1.0	1.1	1.5	1.6	2.0	1.4

SED:	Surfactant treatment	+/- 0.11	(d.f. = 4)
	Leaf position	+/- 0.21	(d.f. = 119)
	Surfactant treatment x Leaf position (at same level of surfactant)	+/- 0.30	(d.f. = 119)
		+/- 0.30	

Table 4.4. Infection scores produced by Erysiphe cruciferarum in relation to surfactant treatment and brassica type (averaged for leaf position).

SURFACTANT TREATMENT	BRASSICA TYPE Swede	Oilseed rape	Brussels sprout	Mean
Untreated	3.4	1.4	0.4	1.7
Treated	1.2	1.4	0.3	1.0
Mean	2.3	1.4	0.4	1.4

SED:	Surfactant treatment	+/- 0.11	(d.f. = 4)
	Brassica type	+/- 0.19	(d.f. = 16
	Surfactant treatment x Brassica type (at same level of surfactant)	+/- 0.24	(d.f. = 16)
		+/- 0.26	

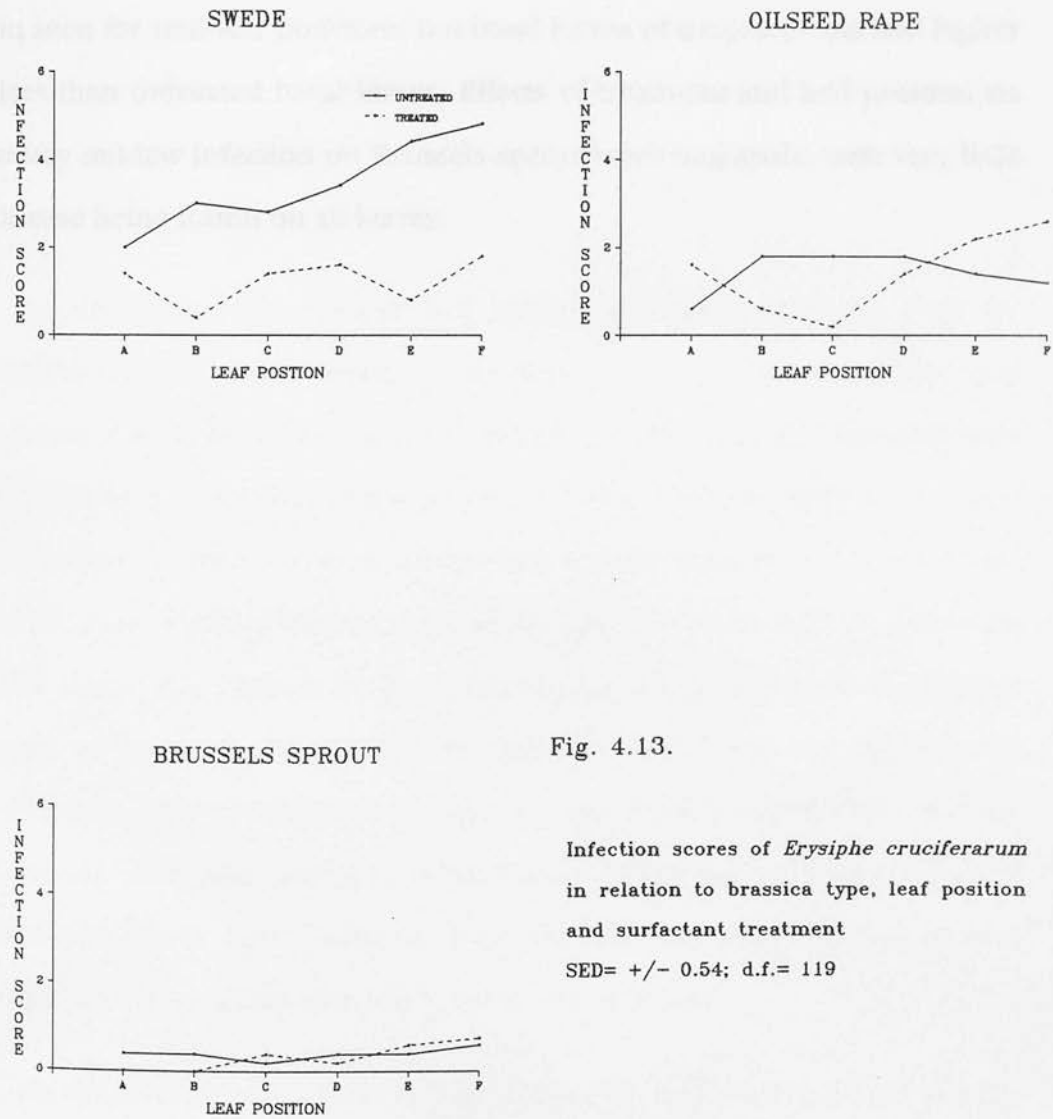


Fig. 4.13.

Infection scores of *Erysiphe cruciferarum* in relation to brassica type, leaf position and surfactant treatment

SED= +/− 0.54; d.f.= 119

The interaction between treatment, brassica and leaf position is illustrated in Fig. 4.13. All leaves of swede, except the very young, were significantly less infected with powdery mildew when treated with surfactant: this effect was most distinct on basal leaves so that leaf position effects were reduced. Significant reductions in powdery mildew on oilseed rape were again seen for mid-leaf positions, but basal leaves of treated plants had higher indices than untreated basal leaves. Effects of treatment and leaf position on powdery mildew infection on Brussels sprout were negligible, with very little of disease being found on all leaves.

4.3.3. Infection of leaf disks by two **Alternaria** *species and* **Erysiphe cruciferarum** *in relation to brassica type, leaf position and environment factors.*

Alternaria brassicicola.

Temperature, light intensity, brassica plant and leaf position each showed a significant main effect on infection by *Alternaria brassicicola,* but interactions between these factors were significant in most cases. (Appendix 4.3).

At the lower temperature and lower light intensity leaves were on average more severely infected (Table 4.5). The effect of temperature was greater at the higher light intensity, whilst the effect of light intensity was significant only at the higher temperature. With respect to brassica plant and leaf position, overall Brussels sprout had a slight tendency to show more infection than swede or oilseed rape, whilst infection was seen to be lowest on middle leaves and highest on basal leaves (Fig. 4.14). However, the greater amount of infection associated with Brussels sprout was evident only on apical leaves. On the basal leaves infection was actually less on this brassica than on the other two. Leaves of swede tended to become progressively more infected as leaves aged, whereas both oilseed rape and Brussels sprout showed their lowest infection levels at mid-leaf positions.

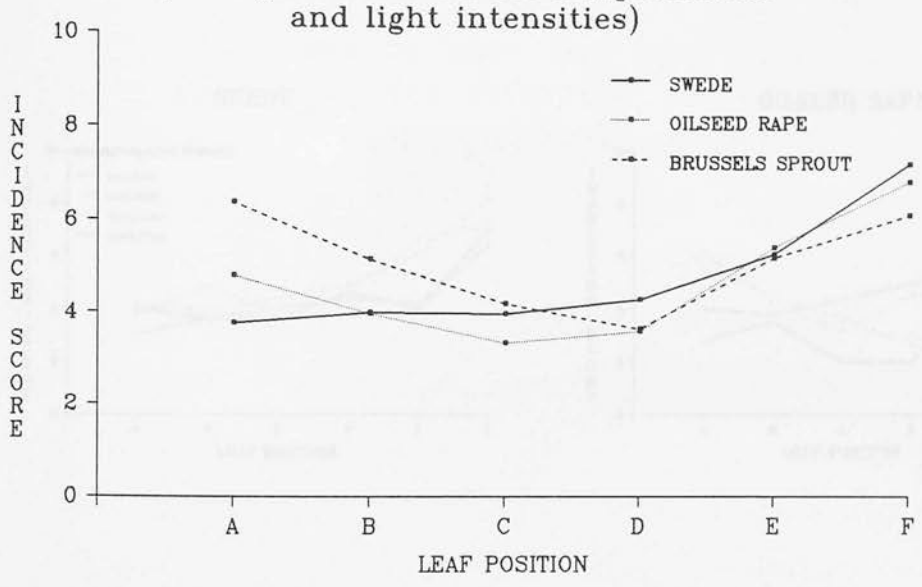
In considering differences between brassicas and environmental regime (Fig 4.15), effects of temperature and light intensity were most evident on Brussels sprout. Reductions in degree of infection were seen when either temperature or light intensity was increased for this brassica, but significant differences in response to light were restricted to the lower temperature. Only

Table 4.5. Infection scores produced by Alternaria brassicicola in relation to temperature and light intensity (averaged for brassica plant and leaf position).

TEMPERATURE	LIGHT INTENSITY Low (4000lux)	High (8000lux)	Mean
Low (12°C)	5.3	5.1	5.2
High (18°C)	4.8	4.1	4.4
Mean	5.1	4.6	4.8

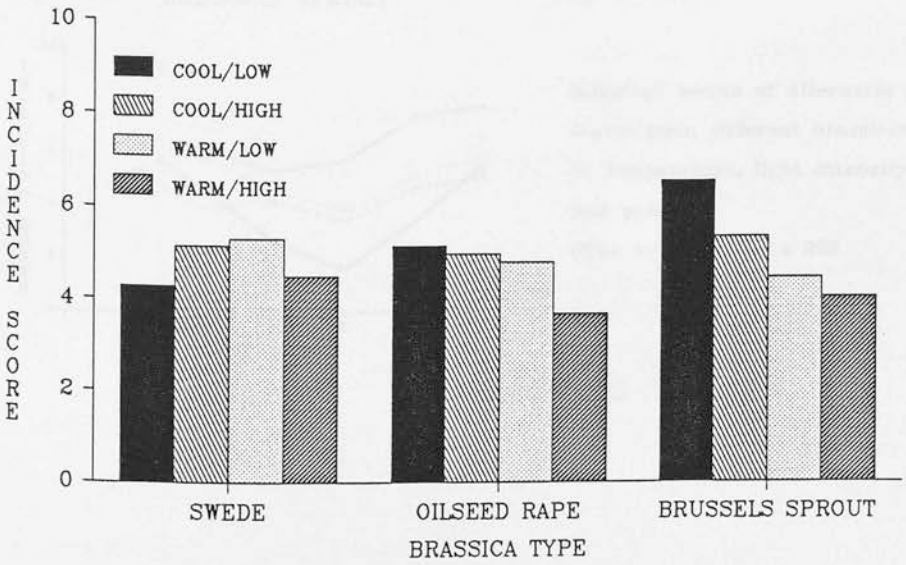
SED:	Temperature	+/- 0.23	(d.f. = 4)
	Light intensity	+/- 0.12	(d.f. = 7)
	Temperature x Light intensity	+/- 0.26	(d.f. = 7)
	(At same level of temperature)	+/- 0.16	

Fig. 4.14. Infection scores of *Alternaria brassicicola* on leaves of different brassicas in relation to leaf position (averaged for different temperatures and light intensities)

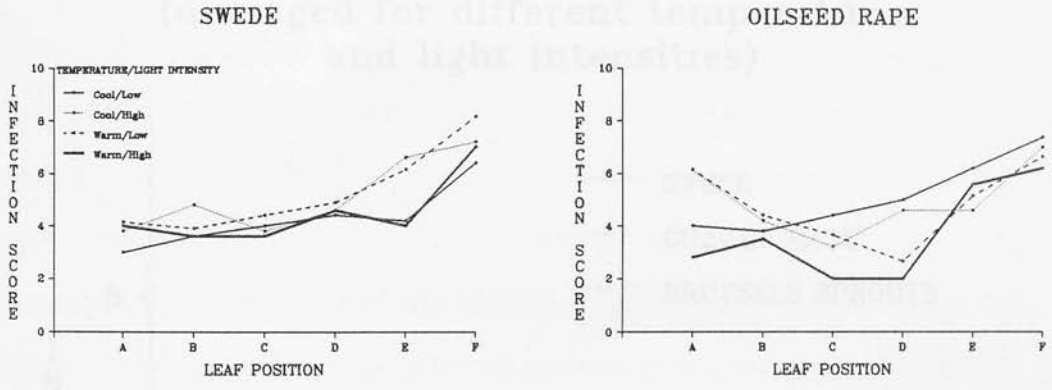


SED= +/− 0.36; d.f.= 223

Fig. 4.15. Infection scores of *Alternaria brassicicola* in relation to brassica type, temperature and light intensity (averaged for different leaf positions)



SED= +/− 0.35; d.f.= 30



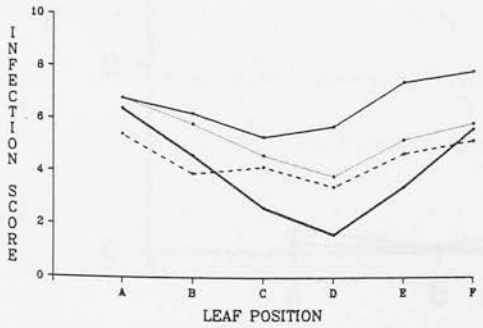
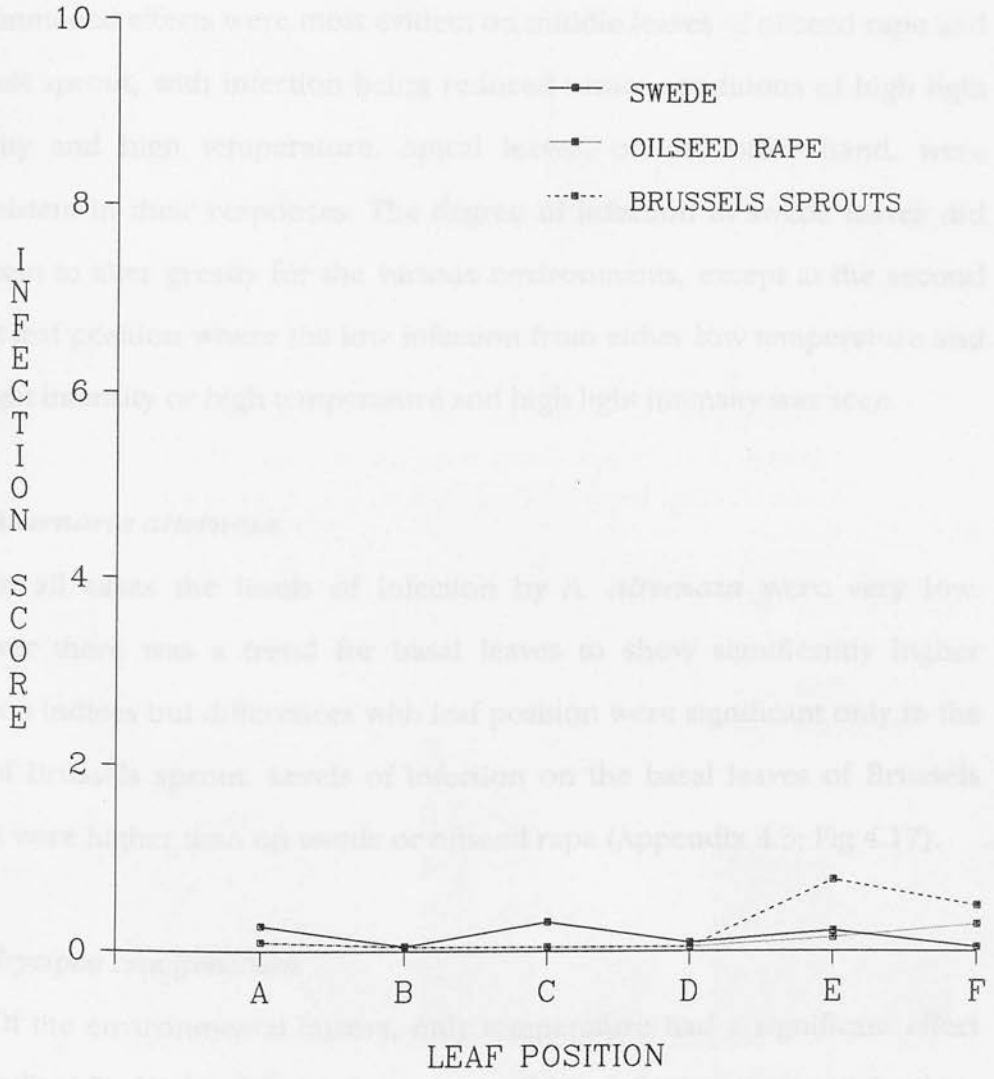


Fig. 4.16.

Infection scores of *Alternaria brassicicola* on leaves from different brassicas in relation to temperature, light intensity and leaf position.

SED= +/- 0.36; d.f.= 223

Fig. 4.17. Infection scores of *Alternaria alternata* in relation to brassica type and leaf position (averaged for different temperatures and light intensities)



SED= +/- 0.18
d.f.= 225

the combination of high temperature and high light intensity appeared to reduce infection significantly in oilseed rape. Swede was unusual in that, while infection levels were relatively low at high temperature and high light intensity they were also low when both temperature and light intensity were low.

The interactions between all four factors are illustrated in Fig 4.16. Environmental effects were most evident on middle leaves of oilseed rape and Brussels sprout, with infection being reduced under conditions of high light intensity and high temperature. Apical leaves, on the other hand, were inconsistent in their responses. The degree of infection in swede leaves did not seem to alter greatly for the various environments, except at the second lowest leaf position where the low infection from either low temperature and low light intensity or high temperature and high light intensity was seen.

Alternaria alternata.

In all cases the levels of infection by *A. alternata* were very low. However there was a trend for basal leaves to show significantly higher infection indices but differences with leaf position were significant only in the case of Brussels sprout. Levels of infection on the basal leaves of Brussels sprout were higher than on swede or oilseed rape (Appendix 4.3; Fig 4.17).

Erysiphe cruciferarum.

Of the environmental factors, only temperature had a significant effect (Appendix 4.3). Higher levels of powdery mildew infection were obtained on plants grown at the low temperature compared with warm conditions: the respective infection scores were 3.1 and 1.5 (sed= ± 0.18; d.f.= 4). In comparing the three brassicas, a significant interaction with temperature and

Fig. 4.19. Infection scores of *Erysiphe cruciferarum* in relation to brassica type and leaf position (averaged for different temperatures and light intensties)

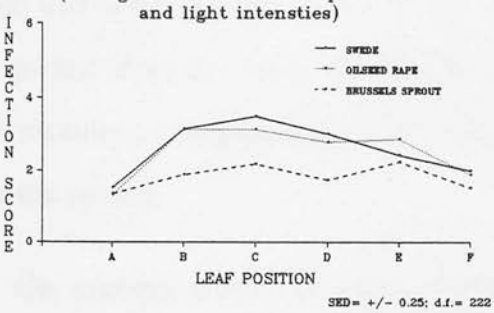


Fig. 4.20. Infection scores of *Erysiphe cruciferarum* in relation to leaf position and temperature (averaged for different brassicas and light intensities)

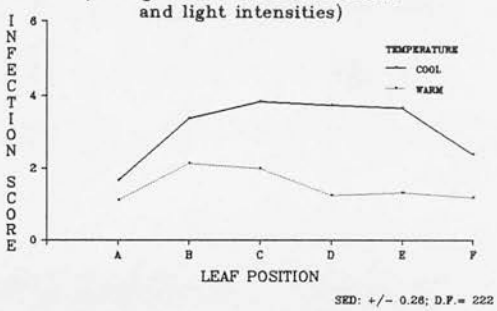
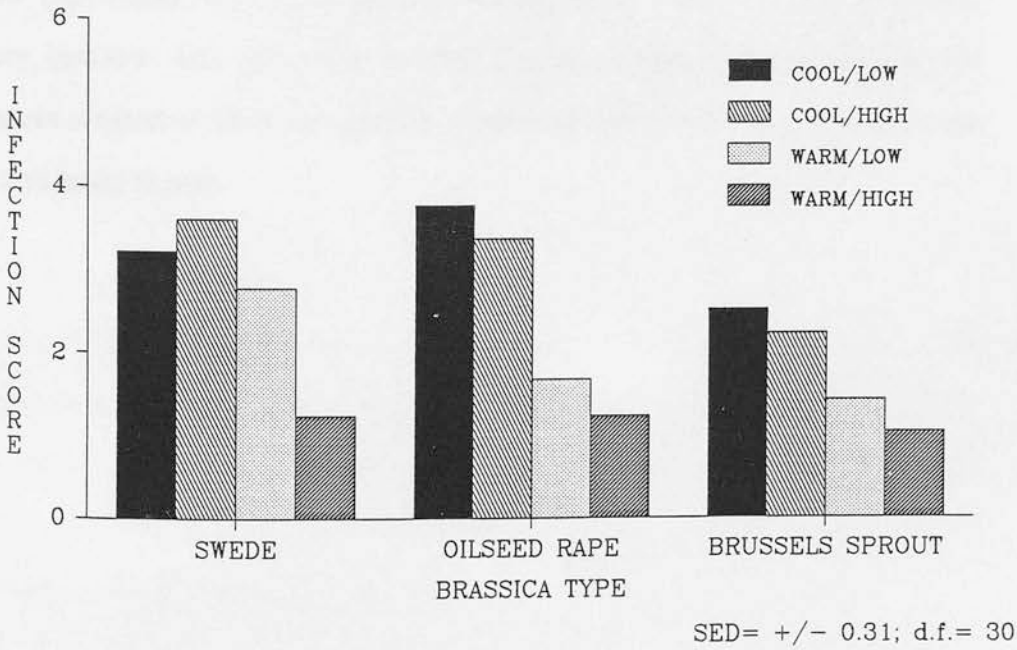


Fig. 4.18. Infection scores of *Erysiphe cruciferarum* in relation to brassica type, temperature and light intensity (averaged for different leaf positions)



light intensity was found (Fig. 4.18). In all cases mildew coverage was significantly reduced on leaves grown at the higher temperature and tended to be reduced with growth at the high light intensity except with swede when grown in cool conditions. Infection indices were similar and at their lowest for all three brassicas at the high temperature and high light intensity. With swede and oilseed rape grown at the low temperature disease development was greater than for Brussels sprout. Where the temperature was high, low light intensity growth gave more infection for swede than for oilseed rape and Brussels sprout.

On average swede and oilseed rape were significantly more infected than Brussels sprout but the differences were significant only at the mid-leaf positions where scores were relatively high (Appendix 4.3; Fig 4.19). Apical and basal leaves of all three brassicas were infected to similar and lower levels. There was a significant interaction between temperature and leaf position (Appendix 4.3) as illustrated in Fig 4.20. Over all leaf positions powdery mildew was favoured by the cooler temperature treatment but differences tended to be most distinct at the mid-leaf positions and least at the apical and basal leaves.

4.3.4. Infection of leaf disks from different leaf positions of different **gemmifera** *mutant lines by two* **Alternaria** *species and* **Erysiphe cruciferarum.**

Alternaria brassicicola.

The infection scores of *gemmifera* mutant leaves are given in Table 4.6 and those for different leaf positions in Table 4.7. Both factors significantly affected disease levels and the interaction between them was also significant (Appendix 4.4). Waxy phenotypes were significantly less infected than glossy phenotypes, intermediate phenotypes having intermediate values (Table 4.6). Averaged over all mutants, sub-apical and mid-leaf positions were least infected and basal leaves the most.

Interactions between the two factors are illustrated in Fig 4.21. The differences between mutant lines were evident at mid-leaf positions, where the severity of disease was lower on waxy phenotypes. Differences in values on apical and basal leaves could not be consistently related to any leaf surface factor.

Alternaria alternata.

Despite low infection rates by *A. alternata*, significant differences were found between different leaf positions and their interaction (Appendix 4.4). As with *A. brassicicola,* waxy phenotypes were less infected compared with glossy phenotypes (Table 4.6), whilst apical leaves and particularly basal leaves were more infected than mid-leaves (Table 4.7).

The interaction between the two factors (Fig 4.22) demonstrated the tendency for apical and basal leaves to give more infection. Differences in

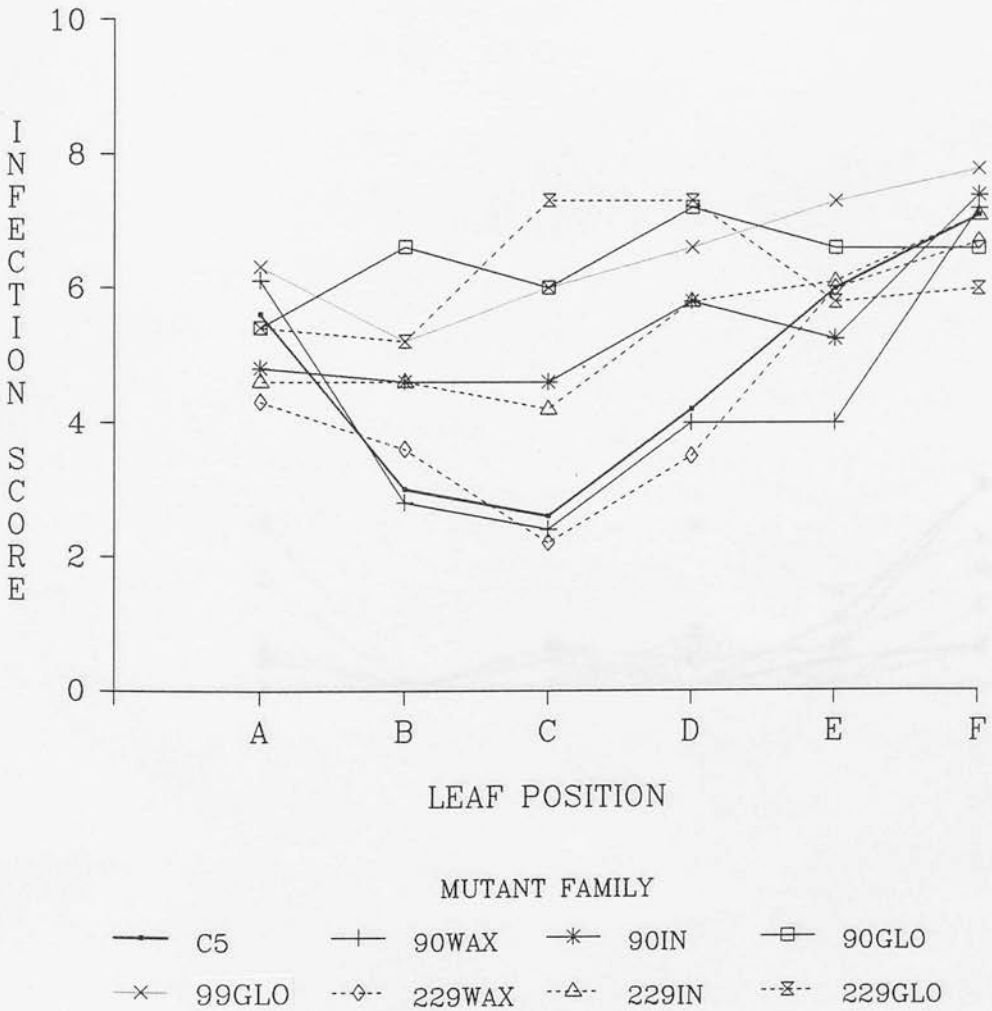
Table 4.6. Infection scores for two Alternaria species and Erysiphe cruciferarum on different gemmifera mutant lines (averaged for leaf position).

MUTANT LINE	*Alternaria brassicicola*	*Alternaria alternata*	*Erysiphe cruciferarum*
C5	4.8	0.2	1.9
90WAX	4.4	0.4	4.1
90IN	5.5	0.7	4.0
90GLO	6.4	0.6	4.0
99GLO	6.5	1.1	2.6
229WAX	4.4	0.2	1.9
229IN	5.4	0.8	2.0
229GLO	6.2	0.8	1.0
SED =	+/- 0.34	+/-0.30	+/- 0.24
d.f.	28	28	28

Table 4.7. Infection scores for two Alternaria species and Erysiphe cruciferarum at different leaf positions (averaged for gemmifera mutant line).

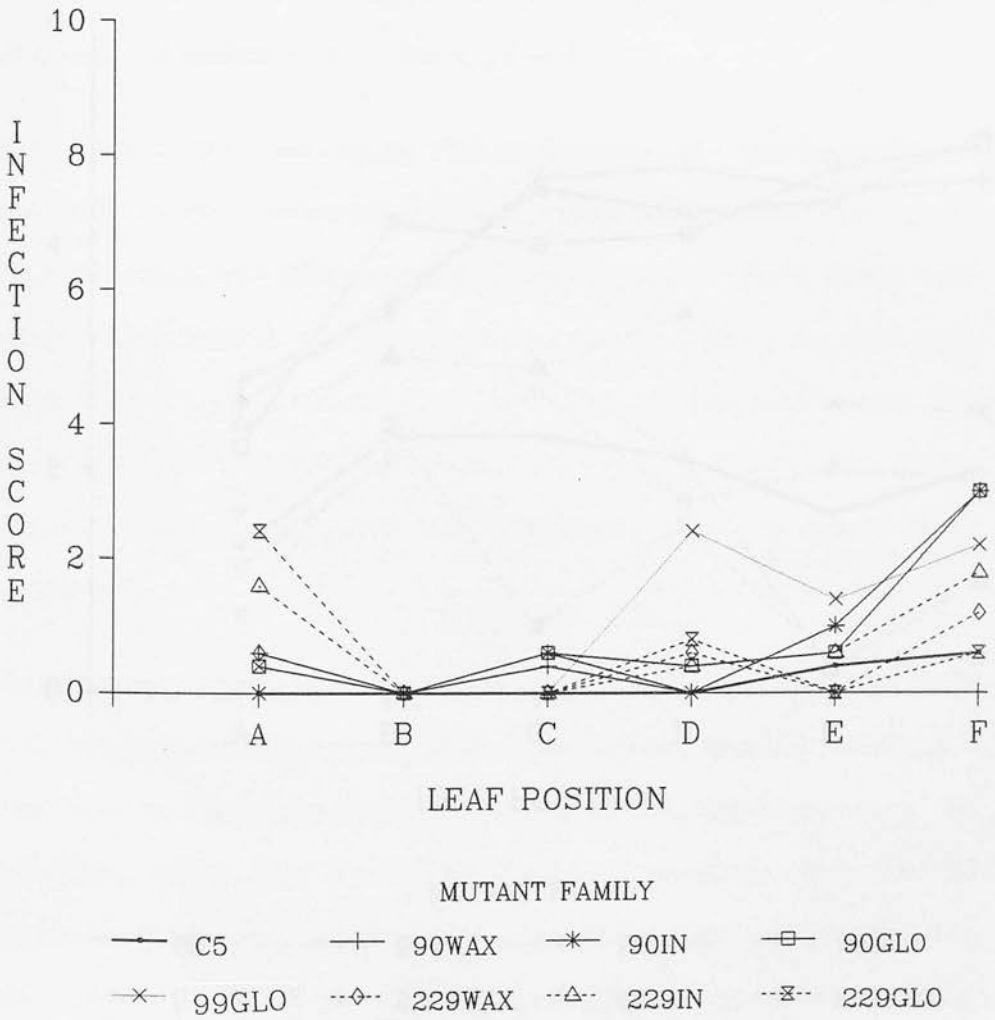
LEAF POSITION	*Alternaria brassicicola*	*Alternaria alternata*	*Erysiphe cruciferarum*
A	5.1	0.8	1.8
B	4.5	0.0	2.9
C	4.4	0.2	3.1
D	5.6	0.6	3.0
E	6.0	0.5	2.5
F	7.0	1.6	2.8
SED =	+/- 0.40	+/-0.25	+/-0.17
d.f.	159	160	157

Fig. 4.21. Infection scores of *Alternaria brassicicola* in relation to *gemmifera* mutant line and leaf position



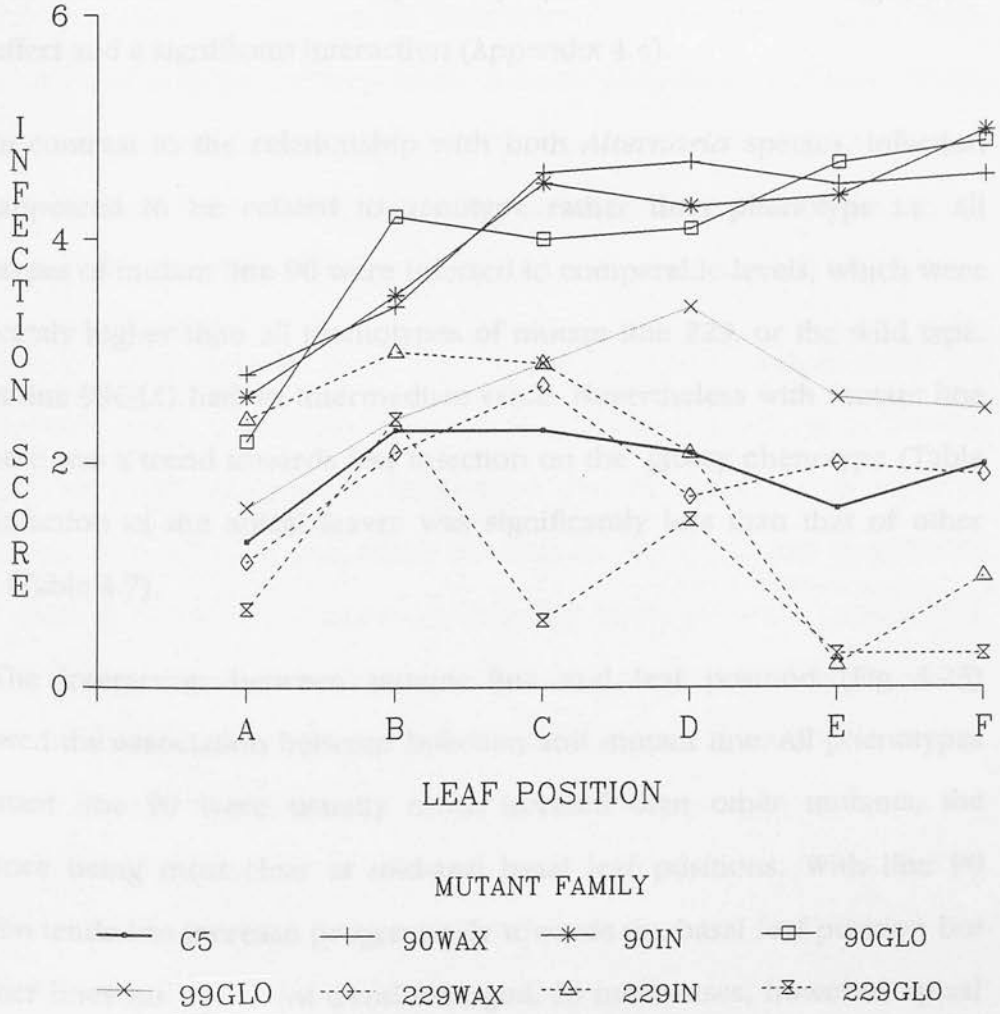
SED= +/− 0.89
d.f.= 159

Fig. 4.22. Infection scores of *Alternaria alternata* in relation to *gemmifera* mutant line and leaf position



SED= +/− 0.70
d.f.= 160

Fig. 4.23. Infection scores of *Erysiphe cruciferarum* in relation to *gemmifera* mutant line and leaf position



SED= +/- 0.50
d.f.= 157

infection between mutant lines were more difficult to clarify due in part to the low indices scored on all mutants but higher infection rates tended to be associated with glossy phenotypes.

Erysiphe cruciferarum.

Scores for powdery mildew in relation to mutant line and leaf position are given in Tables 4.6 and 4.7 respectively. Both factors showed a significant main effect and a significant interaction (Appendix 4.4).

In contrast to the relationship with both *Alternaria* species, infection rates appeared to be related to genotype rather than phenotype i.e. all phenotypes of mutant line 90 were infected to comparable levels, which were significantly higher than all phenotypes of mutant line 229, or the wild type. Mutant line 99GLO had an intermediate value. Nevertheless with mutant line 229 there was a trend towards less infection on the glossy phenotype (Table 4.6). Infection of the apical leaves was significantly less than that of other leaves (Table 4.7).

The interaction between mutant line and leaf position (Fig 4.23) illustrated the association between infection and mutant line. All phenotypes of mutant line 90 were usually more infected than other mutants, the difference being most clear at mid-and basal leaf positions. With line 90 infection tended to increase progressively towards the basal leaf position but for other lines no consistent trend emerged. In most cases, however, apical leaves showed less infection than other leaves, 229GLO being an exception.

4.4. Discussion.

In considering the infection studies as a whole, the experimental factors varied in the extent of their influence, and the pattern of response of the three fungi varied. *Alternaria alternata* produced similar symptoms to *Alternaria brassicicola* in all the experimental treatments, but gave rise to much reduced lesion development, confirming its description as a weak pathogen (Parry, 1990), but associated with several hosts, including brassicas (Holliday, 1989). It has been reported to produce leaf spotting on rape (Vaartnov & Tewari, 1972). In contrast *Erysiphe cruciferarum* showed some distinctive features in its behaviour response to treatment compared with *Alternaria*.

With the three brassica types there seemed to be no obvious trend for one to show more *Alternaria* infection than another. Studying a broader range of brassicas, Prasanna (1984) noted that host species or varieties differed in their infection responses but that the variation was quantitative rather than qualitative in nature. Among the more resistant groups was included Brussels sprout. It was suggested that plants which exhibited greater waxiness of the leaf surface were more resistant, the wax providing a physical barrier which may have impeded infection. A further possible effect of the wax was related to surface tension characteristics of moisture on leaves. Although Brussels sprout did not consistently show less infection than swede or oilseed rape in the present studies, a comparison of Brussels sprout mutant lines, with different leaf surface characteristics showed clearly that glaucous phenotypes were much less infected than glossy types.

A different pattern emerged for the reponse of *Erysiphe cruciferarum* to different hosts. Swede showed most infection and Brussels sprout the least.

The infection studies with *gemmifera* mutant showed clear differences associated with parental lines, but only a slight affect of leaf surface within a parental line. Thus, leaf surface characteristics did seem to play a predominant role in determining the extent of infection by *Alternaria*, while the relationships of *E. cruciferarum* appeared to be based more on the general genetic background of the host.

While swede was found to be more susceptible to powdery mildew than oilseed rape and Brussels sprout, it should be noted that the swede cultivar selected for study, Doon Major, is very susceptible to powdery mildew (Munro, 1985). Other cultivars of this brassica would be expected to have shown much less infection. Thus, the cultivars of the three brassicas used in the experiments are not suggested to be representative of each of the respective brassica groups as a whole.

In the comparison of different leaf surfaces within Brussels sprout mutant lines, in one it was found that a glossy surface gave rise to less powdery mildew infection than a waxy surface. This is in keeping with the results of similar investigations by Munro (1985). He considered that a glossy surface may allow a greater exudation of substances which inhibit *E. cruciferarum* or that water relations are affected in a way which discourages infection.

There was a clear effect of leaf position on infection levels for *Alternaria*, basal leaves invariably being most severely infected. The *Alternaria* disease scores for apical leaves relative to those for middle leaves were less consistent, varying with brassica plant and experiment. One possible reason for the greater fluctuations in behaviour patterns on these leaves is that they would be undergoing developmental changes more rapidly than

leaves towards the basal positions (see, for example, Fig. 3.6). The developmental changes involved could have been important in determining the course of infection. Thus, time of sampling in any one experiment may not have provided upper leaves of exactly equivalent characteristics for each brassica to those from similar positions in another experiment. There was, however, some evidence of an interaction between brassica and leaf position for upper leaves. Apical leaves of swede tended to give less infection than middle leaves and the reverse applying to Brussels sprout. Behaviour patterns on oilseed rape seemed more variable from one experiment to another.

This difference in behaviour between different brassicas may be partly explained by their different rates of emergence and exposure from apical buds. Emerging leaves of *Brassica napus* are exposed relatively rapidly and may undergo changes or possess qualities which reduce infection. Young leaves of *Brassica oleracea* remain covered in the bud longer which may afford them a period of disease escape, but render them vulnerable to attack if they are exposed. Prasanna (1984) also found that infection by *Alternaria brassicae* was greater on older leaves and this was associated with less wax, wax declining with processes of ageing (Skoropad & Tewari, 1977)

The severity of powdery mildew tended to be least on the apical leaves and increased on leaves towards the base of the plant in most cases. This confirms the results of Munro (1985), although Brain (1978) found that resistance increases with increasing leaf age. The present work showed in one instance, where the effects of environmental conditions on plant growth prior to inoculation were investigated, that there was a decline in powdery mildew from middle to basal leaves. It is suggested that the reduced infection on the older leaves was due to the onset of senescence: *E. cruciferarum* is an

obligate biotroph requiring actively living cells to sustain its nutritional requirements. Thus the approach of senescence would adversely affect its development, but prior to the onset of senescence, it may be concluded that susceptibility to mildew increases with leaf age. This agrees with the results of field trials showing that infection of swedes was more severe, the earlier the cultivars were sown (Brain, 1978). Exudation of nutrients on older leaves (Tukey, 1971) may stimulate fungal development. However, Munro (1985) considered that the greater availability of nutrients or stimulatory substances on the surface of older leaves did not wholly account for their increased susceptibility and implied that the functioning of active mechanisms may change with age (Hwang & Heitfuss, 1982).

In attempting to explain the variation in severity of *Alternaria* and *Erysiphe cruciferarum* infection on different hosts and on leaves of different ages, a number of factors may be considered in the context of the present study. These factors include structural and chemical features of the epicuticular wax layer, the wettability of the leaf surface and the presence of leaf exudates at the surface.

With respect to the quantity of epicuticular wax present, this declined rapidly with leaf age during the early phase of leaf emergence. Further decreases were very slight at middle and basal positions on older leaves (Fig. 3.6). The marked changes in levels of disease caused by both *Alternaria* and *Erysiphe cruciferarum* between middle and basal leaves, suggested that the actual amount of wax present had little influence on the course of infection. Wax morphology tended to change with leaf age, crystals becoming degraded and more sparsely distributed on older leaves. Therefore the surface layer of older leaves may have presented a less effective barrier to penetration or the

structural alterations may have resulted in changes in the surface environment with respect to water or nutritional supply.

Absence of marked differences in levels of infection of *E. cruciferarum* on waxy and glossy phenotypes, within the same mutant line, despite their significant differences in wax morphology, would suggest that the structure of the epicuticular wax layer was not important for the development of the pathogen. Nevertheless plants grown at high temperatures showed reduced *E. cruciferarum* infection. High temperature was observed to transform the configuration of wax crystals (Fig. 3.13). *Erysiphe* fungi may require physical stimuli at the leaf surface for their further development (Yang & Ellingboe, 1972). Structural changes such as those induced by high temperature may thus be disadvantageous to the pathogen. On the other hand, the dendritic crystals formed at high temperatures may be effective in excluding hyphal structures from the cuticle proper. It is recognised that high temperature would have altered many plant characters additional to the nature of the leaf surface. Changes in the physiology of plants, not considered here, could have accounted for this effect of temperature on powdery mildew infection. Increased light intensity which gave rise to increased density of wax crystals on the surface had little effect on powdery mildew infection. Alternatively, both temperature and light intensity influenced *Alternaria* infection, less occurring where crystals were denser and showed a dendritic form.

Chemical analysis of the wax layer (Chapter 3) demonstrated that the ketone fraction exhibited fungitoxicity. Despite this, there was no evidence that it had any influence on infection by any of the pathogens tested. In *gemmifera* mutants, ketones were one of the dominant constituents of waxy phenotypes and this might be correlated with lower levels of *Alternaria*

infection. However, quantities of ketones were more or less similar in the three brassica types and showed little variation with leaf position. This contrasts with the marked effect of leaf position on severity of infection by all of the three fungi. The severity of *E. cruciferarum* infection on the different *gemmifera* lines did not seem to relate to the quantities of ketones present in their epicuticular waxes.

The general findings of these studies on infection by *Alternaria* species are in keeping with observations on these and similar "water dependant" pathogens *i.e.* morpholological features of the epicuticular wax affect disease severity (Tewari & Skoropad, 1976; Maddock *et al.*, 1981; Mhunde & Bhowmik, 1985). Higher disease levels are generally found on plants which display little or no "bloom" and this might be associated with water relations at the leaf surface. The increased infection by *Alternaria* on more wettable leaf surfaces was clearly demonstrated when leaves were treated with a surfactant prior to inoculation. Such treatment increased wettability of brassica leaves, especially mid-leaves, which showed the greater degree of water repellancy. This effect was mirrored in the increased infection levels by *Alternaria* following surfactant treatment. Rawlinson *et al.*, (1978) found more light leaf spot (*Pyrenopeziza brassicae*) on oilseed rape which had been previously treated by the herbicide Dalapon (see Chapter 3.4) and concluded that the effects were more likely due to enhanced spread and retention of conidia.

According to Dickenson (1976) free surface water is required for hyphal extension on the surface. Hence on surfaces where droplets remain spherical, such as when the epicuticular wax is in the form of projecting rods or tubes and thereby permitting only a limited area of contact between droplet and surface, surface growth similarly remains limited. Prasanna (1984) observed

differences in disease symptoms produced on oilseed rape leaves which had been inoculated with *A. brassicae* and *A. brassicicola* in intact or dispersed droplets and incubated at varying humidities. High humidities were believed to maintain intact droplets, thus confining growth to the space occupied by the droplet. Dispersed droplets, conversely, allowed greater hyphal extension and increased the potential for parasitisation of the host. Rods or tubes of wax repel water from the surface of the cuticle proper (Martin & Juniper, 1970), droplets being in contact only with the projecting tips of crystals. This presumably reduces the possibility of any infection structure breaching the barrier.

The positive relationship between increased surface wettability and increased *Alternaria* infection, shown in the surfactant experiment was found in other experiments. Thus the reduced infection found on plants that had been grown at high temperature and high light intensity was complemented by the greater water repellancy of leaves grown under these conditions. Likewise the more resistant, waxy phenotypes of *gemmifera* mutants showed a greater water repellancy. In considering leaf age effects, middle leaves were more water repellant than basal leaves and appeared more resistant to *Alternaria* infection. The relationship, however, between wettability and susceptibility was less consistent in comparing apical with middle leaf positions. Once again other factors may also be involved in determining the extent of infection of apical leaves in the case of *B. napus* plants.

The effect of pre-treatment with surfactant on infection by *E. cruciferarum* was the reverse of that on *Alternaria* infection, treatment reducing powdery mildew. Munro (1985) has suggested that infection is

reduced on wettable leaf surfaces. According to Wheeler (1981) where water is retained more readily conidial germination and growth of powdery mildew is delayed. However, there was no straightforward relationship between water repellancy and infection by *E. cruciferarum*. Basal leaves, which showed high wettability, were often most susceptible to infection.

The extent of exudation of electrolytes from leaf tissues to the leaf surface may have influenced fungal development to some extent. Thus, with *Alternaria*, it may be argued that the greater amount of infection on basal leaves and on glossy phenotypes is linked with a greater availability of nutrients at the plant surface (indicated by the permeability experiment in Chapter 3). From these results it is not possible to establish the relative importance of the wettability and permeability characters of the leaf surface. Nevertheless, the surfactant experiment would suggest that the wettability of the leaf surface is at least in part responsible in determining *Alternaria* infection.

In the case of *E. cruciferarum* older leaves showing greater permeability allowed more infection. Munro (1985) thought that this might be partly explained by a greater accessibility of nutrients and stimulatory substances at the plant surface of older leaves. However, glossy leaf phenotypes of Brussels sprout mutant lines showed greater permeability but tended to show similar or slightly less mildew infection, compared with waxy phenotypes. Munro (1985), from similar findings, suggested glossy leaves might allow greater exudation of inhibitory substances. From the present studies there is no evidence to support this, and it is suggested that the most decisive factor determining parasitism of powdery mildew in brassicas is located within the host cells rather than at the plant surface.

The series of experiments in this chapter have attempted to relate the disease development of these pathogens to leaf surface factors. How disease reactions of the pathogen to these features are translated through their growth and development at the surface and early plant responses will now be explored in the following chapter.

CHAPTER 5

DEVELOPMENT OF TWO ALTERNARIA SPECIES AND ERYSIPHE CRUCIFERARUM ON BRASSICA LEAVES WITH DIFFERENT LEAF SURFACE CHARACTERISTICS

5. DEVELOPMENT OF TWO ALTERNARIA SPECIES AND ERYSIPHE CRUCIFERARUM ON BRASSICA LEAVES WITH DIFFERENT LEAF SURFACE CHARACTERISTICS

5.1. Introduction

The events leading up to the successful penetration by a foliar pathogen of its host and the establishment of a symbiotic relationship begin with the arrival of the disease organism on the leaf surface. Fungal propagules achieve contact by a number of means, including dry deposition, transfer by vectors such as insects and wet deposition in rain drops and rain splash. Retention on the surface is complicated by the turbulence and side-slip of air streams, and run off as a result of subsequent rains. In addition, a dense epiphytic microflora may present a mechanical obstruction to the pathogen, preventing it from reaching the surface. These factors have been discussed to some extent in the previous chapter.

Germination is the first crucial event in the life cycle of all fungal pathogens. The majority of spores cannot germinate except in a drop of water or in an atmosphere of 100% humidity, and with decreasing humidity the number of fungi whose spores germinate falls off (Tarr, 1972). Thus, in most cases, the first essential factor for successful germination is a wettable leaf surface. Conn & Tewari (1989) demonstrated the importance of a wettable leaf surface for germination of *Alternaria brassicae* on canola. Wiping canola leaf surfaces which were visibly glaucous significantly increased the rate of germination and the number of germ-tubes produced. The effect was not due

to fungistasis, but considered to be a physical phenomenon whereby the wax directly retarded germination by creating an unfavourable hydrophobic environment and also indirectly by reducing the diffusion of stimulatory leaf exudates to the surface.

Even in an environment which is favourable for germination, spores may still not germinate due to the presence of chemical inhibitors or the absence of stimulatory substances. Dormancy is maintained by natural endogenous inhibitors of germination, compounds which are believed, for example in rust fungi, to combine and inactivate the trigger proteins that initiate the germination process (Trione, 1981). It is only when these inhibitors are removed that germination will occur. The first endogenous germination inhibitor identified was from the uredospores of the bean rust fungus *Uromyces phaseoli*, and was shown to be methly-3,4-dimethoxycinnamate (Trione, 1981). In a number of other rust fungi studied, the inhibitors were a group of similar *cis*-isomers of cinnamic acid derivatives. Endogenous germination inhibitors have since been identified from other fungal genera: quiesone a β-ione compound is a self inhibitor of conidial germination of *Peronospora tabacina*, the organism responsible for downy mildew of tobacco (Leppick, Holloman and Bottomley, 1972). Gloeosporone, a self inhibitor of *Colletotrichum gloeosporioides* germination, has a unique structure compared to other known self inhibitors (Lax, Templeton & Meyer, 1985). It is the only example of a self inhibitor which is the internal γ-lactol of a 4,5-dioxocarboxylic acid.

There are several reports of the occurrence of inhibitors to germination originating from within plant tissue, thereby affording some protection to the host. One of the first accounts of such inhibitors was by Link & Walker (1933), who associated resistance of red and yellow pigmented varieties of onion to

Colletotrichum circinans with higher concentrations of catechol and protocatechuic acid in the scale leaves. These phenolic compounds are released into water present on the surface where they inhibit conidial germination.

Phenolic compounds are responsible for inhibiting germination of pathogens in a wide variety of hosts (Blakeman & Atkinson, 1981). Phenolic substances released from lilac (*Syringa vulgaris*) have been found to inhibit germination of *Alternaria alternata* and *Botrytis cinerea* (Godfrey & Clements, 1978) whilst the presence of gallic acid on Norway maple (*Acer platanoides*) may in part be the cause of low populations of *Cladosporium herbarum* on these leaves (Dix, 1974; 1979). Other groups of compounds active against germination and originating from within the host include several terpenoids, organic acids, and polyacetylenes (Blakeman & Atkinson, 1981).

Alternatively, host plants may release chemical stimulants which initiate germination. Generally the phenomenon is nutritive: host assimilates exuded to the surface as leachates supplement the pathogens own nutrient reserves. Quantitative analysis of leachates show they contain a great variety of materials including mineral salts, free sugars, amino acids, organic acids, pectic substances and plant growth regulators (Tukey, 1971), all of which have the potential for uptake by leaf surface organisms. The levels of macromolecular carbohydrate, fructose and glucose and low molecular weight nitrogen compounds in water droplets on pea endocarps were notably reduced when the droplets were prior inoculated with spores of *Monilinia fruticola* (Smith & Cruickshank, 1984). The fungus required exogenous carbon and nitrogen for germination *in vitro*, thus it was assumed that *Monilinia* gained all required nutrients from leachates in droplets.

There are several examples where chemical signals operate in inducing

germination, probably by counteracting germination inhibitors. Volatile flavour compounds are reported as being active stimulatory moieties in many genera, including *Ustilago, Penicillium, Fusarium* and *Alternaria* (French, 1985). On some occasions the stimulants are components of leachates. Pycnidiospores of *Diaporthe perniciosa* germinate more readily in the presence of *p*-coumarylquinic acid, chlorogenic acid and caffeic acid, all of which are components of apple leachates (Brown & Swinburne, 1978). The pathogen is also stimulated by anthranilic acid, from banana leachate, whilst apple leachates, as well as banana leachate, stimulate germination of *Colletotrichum musae*. Thus these stimulants are believed to be non-specific.

Prior to the penetration process, there is usually a period when differentiation into germ-tubes, mycelium and infection structures occurs, but the principle objective of this growth is often the location of suitable infection sites in order that the pathogen may escape from the adversity of the leaf surface environment. For example an obligate parasite such as *Bremia lactucae* will penetrate the host almost immediately after germination (Cohen, 1981). On the other hand *Septoria nodorum* (a facultative parasite) forms extensive superficial mycelial growth over the leaf surface before penetration. This probably leads to more infection sites and determines the final size of the necrotic area (Baker & Smith, 1978).

Germination of a fungal spore is an extremely complex process involving utilisation of carbohydrate, lipid and amino acid stores, for biosynthesis of nucleic acids, proteins, membranes and cell walls necessary for development. The nutrient demand on the pathogen at this time is great, with the result that the endogenous reserves of the spore may be exhausted before penetration is complete. The developing organism must then depend on an external supply of nutrients, the amount of surface growth determining the extent of the

demand.

Nutrients at the leaf surface are available from a number of sources. Some of these materials originate outside the plant, and are deposited from the atmosphere (Blakeman, 1971). Mineral particles and organic debris can increase infection by *Botrytis cinerea* on strawberry and raspberry fruits, when the organism had been previously in a saprophytic phase (Jarvis, 1962). The presence of pollen on leaf surfaces has been shown to have a significant effect on the infections caused by facultative parasites such as *Alternaria, Fusarium* and *Phoma* (Preece, 1975). However the largest proportion of nutrients arrive at the leaf surface by leaching.

Many fungi are capable of utilising nutrients from leachates directly to maintain germ-tube growth and promote mycelium development. Orellana & Thomas (1962) demonstrated the importance of leachates in infection of castor bean (*Ricinus communis*) by *Botrytis cinerea*. The exudates were found to contain glucose, fructose, glycosides, glutamic acid, aspartic acid, histidine and a tyrosine related compound. It appeared the sugars were influential since high susceptibility was associated with large amounts of surface sugars and *vice versa*. Concentration of sugars within leaves may also be important in determining resisitance to disease. For example during extended periods of darkness, the concentration of sugars in leaves of sugar-beet tissues decreases. This results in a reduced effectiveness of resistance to sporulation of *Peronospora farinosa*. Conversely, spraying leaves with sucrose solution increases resistance (Russell, 1971).

Substrates leached by plants which cannot be utilised by fungal pathogens may become available due to the action of foliar epiphytes. For instance, sucrose, the major cabohydrate leached, is in equal quantities on the

surface with glucose and fructose. The invertase for the degradation reaction is thought to be from phylloplane bacteria.

Although there are a few instances where the phylloplane microflora assists infection, the majority of interactions reported are antagonistic. The commonest form of antagonism is based on nutrient competition. Necrotrophic pathogens such *Septorium nodorum, Botrytis cinerea, Phoma betae* and *Alternaria* species, which require exogenous nutrients for further growth, are antagonised at this stage in development (Fokkema, 1976). Prevention or restriction of growth of pathogenic fungi may result from the activities of hyperparasites. The subject of hyperparasitism has been reviewed by many authors (Fokkema, 1976; 1981; Skidmore, 1976; Kranz, 1981) but basically three types are evident. Necrotrophic and biotrophic interactions are analogous to the relationships observed between plants and fungi, however the third form of hyperparasitism, termed hyphal interference, would seem to occur only when the hyphae from two phylloplane fungi meet, with the result that one succumbs to the other. The interaction may be a form of competition between two residents on the surface. Hyphal interference reactions are the basis of the biological control exerted by *Peniophora gigantea* against *Heterobasidium annosum* (Skidmore, 1976).

Apart from direct competition, parasitism and hyphal interference, the release of antifungal substances as by-products of phylloplane microbial metabolism, may restrict development of fungal pathogens. Many epiphytes are known to produce antibiotics, toxic metabolites or staling substances in culture (Skidmore, 1976). These include strains of *Bacillus, Pseudomonas, Chromobacterium and Agrobacterium* (Blakeman & Brodie, 1976), in addition to phylloplane fungi such as *Aureobasidium, Alternaria, Botrytis*

and Helminthosporium (Skidmore, 1976). The concentrations in which such substances are released is largely unknown, therefore their significance in the antagonistic interactions which have been studied is questionable.

The location of a suitable infection site by a germ-tube is often considered to be the result of chemotropism i.e. a positive or negative response to an external source of chemicals, or contact tropism (thigmotropism), a response to large, insoluble molecules on the surface, including polymers of the cuticle. Orientation of a germ-tube towards an infection site is most pronounced in rust fungi, nevertheless it is evident that the topography of the leaf influences the direction in which hyphae grow in other genera.

Hyphal growth on the surface frequently follows the lines of junctions of underlying cells and ends with appressorium formation (Wynn & Staples, 1981). Despite the view that appressoria develop indubitably as the conclusion of germination (Emmett & Parberry, 1975), they have been shown to mature more effectively on plant surfaces than inert substrates such as glass (Parberry & Blakeman, 1978). The anticlinal wall areas are selected by a wide range of fungal genera for formation of appressoria. It is known the cuticle is diminished in these regions (Kirkwood, 1972) and exudates collect in the crevices created by the cell junctions (Dodman, 1979). Hence they may be more susceptible to fungal attack. Fungi which most consistently display this activity are species of *Erysiphe* (Preece, Barnes & Bayley, 1967; Russo & Bushnell, 1989), *Peronospora* (Preece, Barnes & Bayley, 1967), *Colletotrichum* (Lapp & Skoropad, 1978), *Bremia* (Sargent, Tommerup & Ingram, 1973) and *Septoria* (Baker & Smith, 1978).

The sensing of suitable infection points for correct alignment of

appressoria is a critical step in the pre-penetration process. The signals involved in the induction have been shown in some instances to be chemical in origin (Parberry & Blakeman, 1978; Brown & Swinburne, 1978) but are more often related to the physical nature of the plant surface. Whilst in many cases simple contact alone with the surface is sufficient to activate appressorium formation (Staples & Hoch, 1987), in others more subtle stimuli are required. In the bean rust fungus, *Uromyces appendiculatus,* a ridge elevated 0.5 μm above the surface induces the development of appressoria following germination of uredospores. Such ridges are found on the diaphanous lip of the guard cell. The pathogen being a stomatal-penetrating species thus is capable of detecting appropriate entry sites (Hoch & Staples, 1987).

The distribution of wax bodies on the surface is regularly quoted as the important stimulus of appressorial differentiation. From the appearance of malformed appressoria on dewaxed barley cuticles, reconstituted wax or on plants with wax mutations, Yang & Ellingboe (1972) concluded that *Erysiphe graminis* required an unaltered wax for formation of mature appressoria. However work by Nicholson, Yoshioka, Yamaoka & Kunoh (1988) implied that appressoria could be formed normally in response to waxes which had been previously degraded by an exudate from the ungerminated conidia. Carver & Thomas (1990) confirmed this when they discovered appressoria of *E. graminis* developed similarly on intact oat leaves and leaves which had been stripped of their waxes. It seems likely then that some other, presently unknown, component of the cuticle is significant in normal germling development.

Following a period of extra-matrical development the next critical phase is penetration which in many cases occurs directly through the cuticle. This

mode of entry is of primary interest in these studies. The contribution of physical properties of the cuticle to overall resistance to penetration has been indicated in Chapter 3.1.

Fungal penetration of the cuticle has been examined by many workers (McKeen, 1974; Aist, 1976; Kolattukudy, 1985). For many years penetration was believed to be a truly physical phenomenon, forces required being provided by high osmotic pressures generated in the appressorium. Appressoria are well suited for mechanical breaching of host barriers. Their attachment to the surface by a deposit of mucilaginous material and formation of a fine infection thread, allows concentration of applied force in a restricted area (Aist, 1976). Indeed electron microscopy studies reveal inward depressions at the point of penetration (Aist, 1976).

Yet on many occasions there is a lack of depressions and the cuticle appears "digested". Recent evidence conclusively proves that chemical degradation by a cuticle destroying enzyme is involved. Certain fungi can grow on cutin as a sole source of carbon. Purdy & Kolattukudy (1973) showed that the extracellular fluid from such cultures grown on radioactive cutin, catalysed the release of all types of monomers from cutin, and that they were approximately in the same proportion as that present in the polymer, suggesting production of an extracellular cutin degrading enzyme.

Cutinase was first isolated in homogeneous form from the extracellular fluid of cutin grown *Fusarium solani* f. sp. *pisi* (Purdy & Kolatukudy, 1975). The enzyme is a single peptide serine hydrolase containing the characteristic triad normally associated with active serine: a serine hydroxly group, a carboxly group and an imidazole group of histidine (Kolattukudy, 1985). Other characteristic features of the amino acid composition include the

presence of one methionine, one histidine and one tryptophan per molecule (Kolattukudy, 1985).

Cutinase has several amino acids not hitherto found in other proteins. These are not involved in the activity of the enzyme, but are structural features which give the enzyme properties of great stability. The molecule is resistant to the effects of proteolytic enzymes such as trypsin, chymotrypsin and elastin (Kolattukudy & Köller, 1983), perhaps reflecting its extracellular nature.

Both the hydrolysis of polymers to oligomers and hydrolysis of oligomers to monomers are catalysed by cutinase. The enzyme shows specificity for primary alcohol esters (Kolattukudy & Köller, 1983), the dominant linkages found in cutin. All fungal cutinases thus far examined have similar structural and catalytic properties (Kolattukudy, 1985: Köller & Parker, 1989; Abergel, Martinez, Fontecilla-Camps, Cambillau, de Geus & Lauwereys, 1990; Trail & Köller, 1990).

The most convincing proof for the involvement of cutinase in fungal penetration was presented by Shayku, Soliday & Kolattukudy (1977) and Köller, Allan & Kolattukudy (1982) in their work with *F. solani* f. sp. *pisi* and its host *Pisum sativum*. Ferritin-conjugated antibodies, prepared against cutinase, forms a complex with the enzyme and this can be visualised with electron microscopy. The complex was indeed observed when the anti-cutinase was applied to the penetrating area. Since the anti-sera prevented infection only when an intact barrier was present, the cutinase was considered necessary for penetration. Control sera had no effect on infection.

A similar approach demonstrated cutinase is essential for penetration of papaya fruits by *Colletotrichum gloeosporoides* (Dickman, Patil & Kolattukudy, 1982). Cutinaseless mutants are able to infect only after the

cuticle is mechanically breached. Further, a species of *Mycosphaerella*, which relies on wounds for penetration, infects papaya fruits when the surface is pretreated with purified cutinase from *C. gloeosporoides*.

Enzymic hydrolysis of the cuticle is now well established as the means by which at least some fungi gain access through the surface barrier (Wynn & Staples, 1981; Trail & Köller, 1990). However the relative importance of enzymic degradation and physical force probably varies with host-pathogen relationship. In the cases where the cuticle acts as a mechanical barrier, penetration is probably mechanical but may still be facilitated by localised enzymic activity. The ability to penetrate the cuticle with relative ease is probably due to enzymatic degradation of the cutin matrix. The degradation of the cuticle and epicuticular wax before penetration, at least in *Erysiphe graminis,* is best described as "preparation of the infection court" (Nicholson *et al.,* 1988).

Following cuticle penetration some pathogens adopt a sub-cuticular lifestyle, relying on extracellular enzymes and toxins to release substrates. Further invasion of the host requires repeated contact and breaching of host cell walls. Plant pathogens possess an arsenal of cell wall degrading enzymes (CWDEs), corresponding to most of the glycosidic linkages present in the wall. CWDEs are ubiquitous amongst plant pathogens, but the quantities in which they are secreted is specifically determined.

Of primary importance in wall degradation of dicotyledonous hosts are the *endo*-pectic enzymes, especially *endo*-polygalacturonidases since they are the first CWDEs produced (Cooper & Wood, 1975). This contrasts with cereal pathogens where pectic enzymes are characteristically absent, perhaps in adaptation to the low galacturonide content of cereal cell walls (Cooper,

Longman, Campbell, Henry & Lees, 1988).

Endo-pectic enzymes attack the internal regions of the rhamnogalacturon backbone of pectin, thereby effecting disruption of the wall matrix. If large quantities of *endo*-pectic enzymes are produced, as in necrotrophic reactions, then the structure is weakened to the point when it can no longer contain the pressure exerted by the protoplast, resulting in a damaged plasmalemma and cell collapse (Cooper, 1983). In addition degradation of the middle lamella leads to cell separation, and with the combined effects complete tissue maceration ensues.

The action of *endo*-pectic enzymes is frequently stated as being centrally important in the pathogenicity of certain necrotrophic organisms (Cooper, 1983). However Durrands & Cooper (1988) demonstrated that *endo*-pectic enzymes were not vital for invasion or nutrition of the vascular wilt pathogen *Verticillium albo-atrum* but were virulence factors involved in symptom expression. Certainly *endo*-pectic enzymes are prerequisites to attack by enzymes degrading other cell wall polymers (Cooper, 1983). Degradation of the wall matrix probably permits access of CDWEs to less exposed substrates such as hemicelluloses and cellulose.

Cellulose degradation occurs less commonly and requires the complex of enzymes originally designated by Reese (1977) as C_1, C_x and cellobiase. C_1 is the only complex capable of attacking native cellulose and is rare in plant pathogens. It operates by rendering crystalline cellulose open to attack by C_x (an *endo*-β-1,4-glucanase) which converts the loosened chains into cellobiose oligomers. Conversion into glucose is complete after degradation of cellobiose by cellobiase (β-glucosidase).

Necrotrophic pathogenesis is often associated with profound wall changes. In contrast, the nature of biotrophic interactions dictates minimum host wall degradation. CDWEs are, surprisingly, produced by obligate parasites, but in very small amounts and in partly bound forms preventing diffusion through the host wall matrix (Cooper, 1983). *Endo*-polygalacturonidases, minute amounts of which have significant effects on wall structure appear to be absent from the battery of CWDEs of biotrophs (Baker, Aist & Bateman, 1980).

CWDEs are believed to be involved in recognition and disease resistance. Davis, Darvil and Albersheim (1984) discovered that an α-1,4-*endo*-polygalacturonase is active in inducing phytoalexin accumulation in soybean. The enzyme cleaved an oligogalacturonide from the cell wall, which acted as an elicitor to phytoalexin synthesis. Elicitors formed in this way have since been discovered in a multitude of other plants, suggesting that at least some incompatibility signals are located in the matrix of the cell wall (Albersheim & Darvil, 1985).

The cell wall is not a static structure during penetration but is the first component of the hosts active defence system. Attempted fungal penetrations commonly result in thickenings on the inner surfaces of cell walls. These deposits are known as papillae, callosities, lignitubers or calli (Aist & Williams, 1971; Ride, 1983).

Papillae form between the host cell wall and plasmalemma at a point directly opposite the fungal penetration peg (Aist, 1976). If development of the fungus is delayed at an encounter site, the depositions are usually hemispherical (Aist & Williams, 1971), whereas if development is in advanced stages, the papillae may assume the shape of the invading moiety and thus

form an encasement (Aist, 1976).

Normally two principle areas are observed when viewing under bright field microscopy (Aist, 1976; Sherwood & Vance, 1976). The outer area (cover) is continuous with the inner layer of the cell wall, and forms a thin layer between the host plasmalemma and the inner area (core). The core is the main body of the papilla and many materials including lignin, callose, suberin, silicon, pectin and inorganic elements may become deposited within this region as strengthening components (Sherwood & Vance, 1976; Vance, Kirk & Sherwood, 1980; Koga, Zeyen, Bushnell & Ahlstrand, 1988; Russo & Bushnell, 1989).

A common response often associated with papilla formation is an alteration to the adjacent epidermal wall to produce a disk shaped area, the halo. In addition a re-orientation of the cytoplasm towards the point of penetration usually precedes the appearance of the papilla (Bushnell & Berquist, 1975). The aggregation is considered to deposit necessary precursors for the synthesis of the papilla (Aist, 1976). In fact papillae contain a variety of membrane fragments, vesicles or organelles, which are believed to be the result of their entrapment during formation of papillae.

The most popular theory regarding the function of papillae is that of an active barrier formation in response to penetration (Vance & Sherwood, 1977; Vance, Kirk & Sherwood, 1980; Smart, Aist & Israel, 1987). Many instances have been reported where parasitic structures have been impeded by papillae, correlations evident between incidence, morphology and size of papillae with penetration failure and not establishment of infection. The production of lignified papillae underneath sites of attempted penetration by the pathogen *Helminthosporium cateranium* and the non-pathogen *Botrytis cinerea* were

demonstrated in reedcanary grass (Vance & Sherwood, 1976). Penetration occurred through less than 2% of papillae, and only through those that were poorly developed. The workers therefore assumed that papilla formation was a dynamic response of the plant containing the fungus at the penetration site.

The failure of *B. cinerea* and other non-pathogenic fungi to penetrate unwounded wheat leaves was associated with formation of papillae beneath appressoria, and alterations in surrounding epidermal cell walls (Ride & Pearce, 1979). Lignification was apparent at early stages in both papillae and halos, the latter being thought to slow down the penetration process until the papilla had fully formed, or to prevent growth of the penetration peg laterally along the wall. It was further demonstrated (Bird & Ride, 1981) that numbers of lignified papillae at any one time did not explain degree of resistance, but reflected the extent of fungal colonisation.

More recently Russo & Bushnell (1989) proved that papillae and their composition are dissimilar from general wound plugs. Insertion of a microneedle at a typical point of attack on barley leaves led to an encasement structure around the tip of the needle within the epidermal cells. Similar responses were also seen to ingress by penetration hyphae of *Erysiphe graminis*, but histochemical tests demonstrated that wound plugs contain cellulose and pectin whereas the fungal-induced papillae contained phenols and basic staining material. This suggests that *de novo* synthesis of materials for papillae formation is dependent on stimuli from invading fungi.

The experimental work of this section is concerned with surface behaviour and early events following the penetration process of the two *Alternaria* species and *Erysiphe cruciferarum* on leaves of the three brassicas, in relation to leaf position, and of the *gemmifera* mutants. A study was also

made of the behaviour of *Erysiphe cruciferarum* on a non-host, barley, to compare with growth on swede, as a compatible host, with *Erysiphe graminis* also included in this last work as a non-pathogen of brassicas.

5.2. Materials and methods

For all experimental studies the preparation of brassica plant material and of *Alternaria* inoculum were as described in Chapter 2.4. The inoculum of *Alternaria* was applied to leaf disks (see Chapter 4.2) as 0.2 ml droplets from a sterile, polypropylene syringe. Inoculum preparation and application of *Erysiphe cruciferarum* followed the procedure given in Chapter 2.4. In the final experiment, non-host was represented by barley cv. Golden Promise and non-pathogen by *Erysiphe graminis.* Barley leaf segments were obtained from 4 week old plants grown under conditions similar to those used for brassica material. Conidia of *Erysiphe graminis* f.sp. *hordei* were obtained from infected barley leaf segments, maintained on benzimidazole agar in a Gallenkamp incubator at 18°C with a 12 hour daylength. Conidia were applied using the method for *Erysiphe cruciferarum* inoculation. All leaf disks and segments were incubated in conditions similar to those specified in Chapter 4.2. and for 36 hours.

Specimens were prepared for observation by decolourising in 200% w/v chloral hydrate then double stained by either aniline blue/trypan blue for fluorescence microscopy or toluidine blue/trypan blue for light microscopy (Appendix 5.1). Specimens were mounted in the staining solution for fluorescence microscopy or buffer solution (Appendix 5.1) for light microscopy.

Fluorescent and light microscopical observations were made with a Leitz Ortholux Fluorescent Microscope fitted with TK400 and TK510 dichroic mirrors, UG (280-420 nm) and BG12 (320-500 nm) excitation filters and K460 (410 nm) and K490 (450 nm) suppression filters. SEM observations served to

complement light and fluorescent microscopy in illustrating behaviour of pathogens on the various surfaces. The preparation and examination procedures are described in Chapter 2.5.

A series of five experimental studies were carried out and are considered under the following headings:

5.2.1. Leaf surface development and early penetration events of two **Alternaria** *species in relation to brassica plant and leaf position.*

Both *Alternaria brassicicola* and *Alternaria alternata* were examined on disks (14 mm diameter) of leaves from apical, middle and basal positions, removed from each of five plants of the three brassica cultivars. The experiment was arranged in a randomised block lay-out with five replicates, using a split-plot design, where brassica plant was main plot and leaf position sub-plot.

5.2.2. Leaf surface development and early penetration events of two **Alternaria** *species in relation to* **gemmifera** *mutant line.*

Both *Alternaria brassicicola* and *Alternaria alternata* were examined on disks (14 mm diameter), removed from leaves of the middle position of different *gemmifera* mutants and the wild type. These were arranged in a randomised block design with five replicates.

For both studies on the behaviour of *Alternaria* species, the following parameters were quantified on 25 conidia selected at random from each disk:

1. Gemination rate (as a % of spores).
2. Germ-tube number (averaged for number of germinated spores).

3. Primary germ tube length (averaged for number of germinated spores, using a graticule eyepiece micrometer).

4. Branching of the primary germ-tube (averaged for number of germinated spores).

5. Axis divergence of primary germ-tube (averaged for germinated spores).

6. Number of vesicles on the primary germ-tube (averaged for germinated spores).

7. Number of terminal vesicles (as a % of germinated spores).

8. Location at which growth terminates - either anticlinal wall, periclinal wall or the guard cell area (as a % of germinated spores).

9. Presence of sub-cuticular hyphae (as a % germinated spores).

10. Normal growth or distorted growth of sub-cuticular hyphae either proximal or distal to the point of penetration (as a % of sub-cuticular hyphae).

11. Host cell reaction - either none, localised fluorescence or necrotic browning and associated with or without a sub-cuticular hypha (as a % of germinated spores).

5.2.3. *Leaf surface behaviour and early penetration events of* **Erysiphe cruciferarum** *in relation to brassica plant and leaf position.*

Disks (14 mm diameter) of leaves from apical, middle and basal positions were removed from each of five plants of the three brassica cultivars. The experiment used a split-plot design, where brassica plant was main plot and leaf position sub-plot.

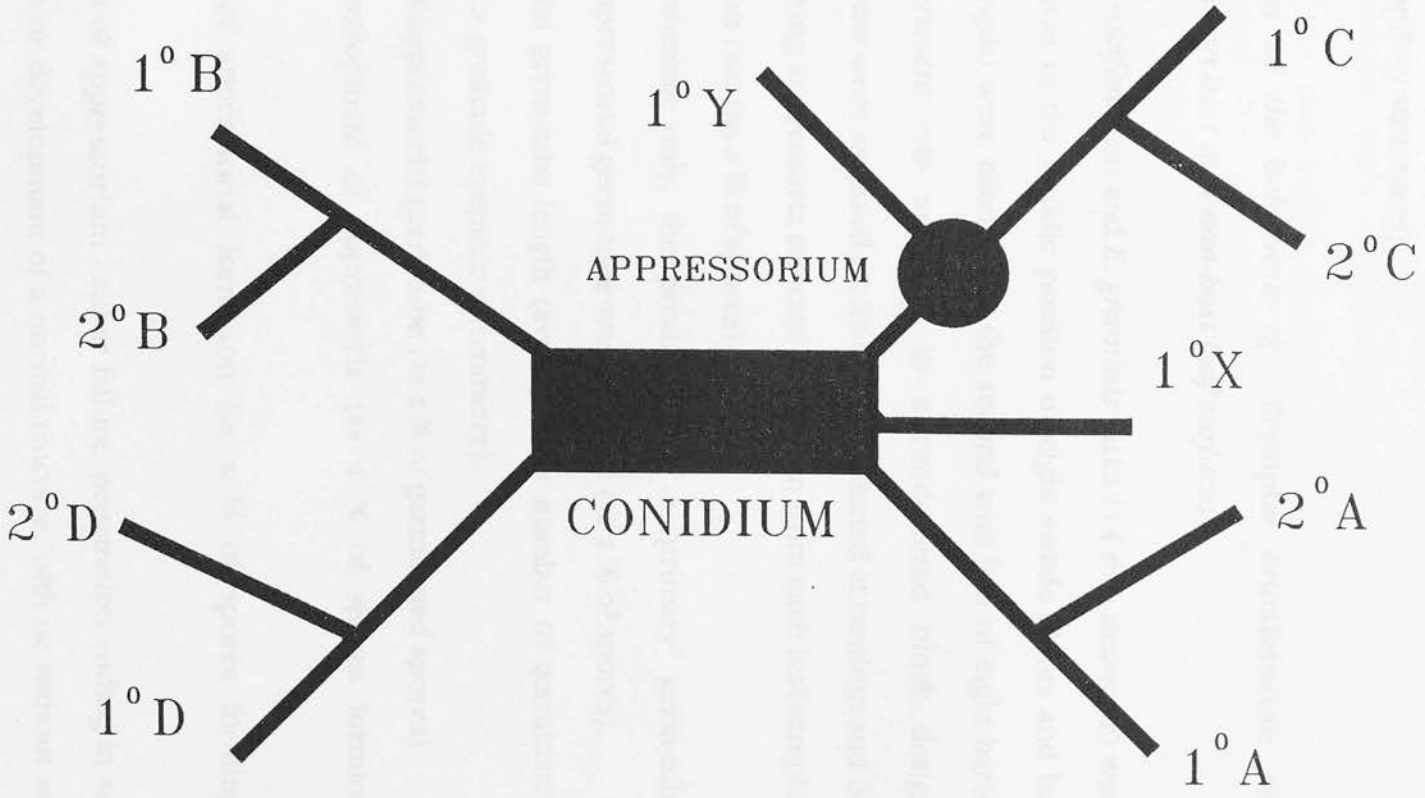
5.2.4. Leaf surface behaviour and early penetration events of **Erysiphe cruciferarum** *in relation to* **gemmifera** *mutant line.*

Disks (14 mm diameter) were removed from leaves of the middle position of different *gemmifera* mutants and the wild type. The experiment was arranged in a randomised block design with five replicates.

For both experiments on the behaviour of *E. cruciferarum*, the following parameters were quantified on 25 conidia selected at random on each leaf disk:

1. Germination rates (as a % of spores).
2. Germ-tube length (averaged for germinated spores, using a graticule eyepiece micrometer).
3. Numbers of appressoria formed (as a % of germinated spores).
4. Size of appressoria (averaged for numbers of spores forming appressoria, and estimated from general size and number of lobes formed, on a scale 1-6).
5. Location of appressoria (either periclinal, anticlinal or guard cell area, as a % of those spores forming appressoria).
6. Numbers of appressoria aborting after attempted penetration (as a % of germinated spores).
7. Numbers of conidia forming secondary structures *i.e.* hyphal growth beyond appressorial stage (as a % of germinated spores).
8. Host reaction- either no reaction or formation of a papilla, with or without an associated secondary structures (as a % of germinated spores).
9. Numbers of germ-tubes and frequency of their positions on the conidium (see Fig. 5.1; as a % of spores forming secondary structures).
10. Numbers of branches and their positions on the germ-tubes (see Fig.

Fig 5.1. Nomenclature of early hyphal development of Erysiphe cruciferarum



5.1; as a % of spores forming secondary structures).

11. For experiment 5.2.4. only, the numbers of conidial initials and their positions on germ-tubes and branches was assessed (as a % of spores forming secondary structures).

5.2.5. Comparison of the behaviour of **Erysiphe cruciferarum** *and* **Erysiphe graminis** *on host and non-host leaf surfaces.*

For both *E. cruciferarum* and *E. graminis* disks (14 mm diameter) were removed from leaves of the middle position of eight swede plants and leaf segments (2cm length) were taken from the second true leaf of eight barley plants. The experiment was arranged in a randomised block design. Parameters 1-5 below were assessed on 25 conidia selected at random and 6-9 on 15 conidia forming appressoria selected at random from each leaf sample.

1. Germination rate (as a % of spores).

2. For *E. graminis* only, the production of a "primary" germ-tube without an appressorial germ-tube was assessed (as a % of spores).

3. Appressorial germ-tube length (averaged for number of germinated spores, using a graticule eyepiece micrometer).

4. Abortion of appressorial germ-tube (as a % of germinated spores)

5.Normal development of appressoria (as a % of spores forming appressoria).

6. Location of appressorial formation (as a % of spores forming appressoria).

7. Penetration of appressorium - either failure, penetration ending in an encapsulation or development of a normal stucture, with or without an associated papilla (as a % of spores forming appressoria).

8. Host cell reaction, either none or degenerated cells (depicted as

bright,flecking fluorescence) (as a % of conidia forming appressoria).

9. Formation of secondary structures (as a % of conidia forming appressoria).

Where percentages were calculated for a parameter, an angular transformation of the data before analysis of variance was carried out. However transformed data did not show any major differences from untransformed data, thus the latter only has been used in presenting the results

Alternaria brassicicola and *Alternaria alternata.*

From observations made 36 hours after inoculation, both *Alternaria brassicicola* and *Alternaria alternata* were found toproducea primary germ-tube, with an occasional secondary and tertiary germ-tube. The primary germ-tube grew for a measured distance on the surface, during which time branching, digressions from a straight course (axis divergence) and vesicle formation could occur (Fig 5.2). Vesicles were seen to be swellings of the germ-tube hypha and arose either in an intercalary or in a terminal position (Figs 5.3 and 5.4). The germ-tube completed growth at a specified site, being the periclinal wall, anticlinal wall or guard cell area (Fig. 5.5). It is assumed that an attempted penetration then took place. Not all growth gave rise to successful penetration especially with *A. alternata* (Figs 5.4 and 5.5).

Successful penetrations were indicated by fluorescing sub-cuticular hyphae originating below the site of penetration. These either grew on normally (Fig. 5.6) or differentiated into folded, swollen structures proximal or distal to the penetration point (Figs 5.7 and 5.8). Sub-cuticular hyphae frequently followed the middle lamella region of the epidermal cells walls (Fig. 5.9).

The host cells reacted in several ways to penetrating hyphae. Besides showing no reaction, areas of localised fluorescence (termed papillae) were sometimes observed (Figs 5.8 and 5.9) and associated with encroachment by hyphae upon the epidermal cell walls (Fig. 5.6). Alternatively, cells in the locality of penetration became necrotic (Fig 5.10), depicted as browning and loss of ability to fluoresce. They were usually surrounded by cells which had

Fig. 5.2. Development of *Alternaria brassicicola* on middle leaf of swede. Stained with trypan blue (x 100 magnification).

Fig. 5.3. SEM of *Alternaria brassicicola* germ-tube on leaf of 99GLO showing intercalary and terminal vesicle.

Fig 5.4. *Alternaria alternata* on mid-leaf of swede, showing vesicle terminating over anticlinal wall and papilla formation by host. Stained with toluidine blue/trypan blue (x 400 magnification).

Fig. 5.5. *Alternaria alternata* on mid-leaf of swede, showing intercalary and terminal vesicle and papilla formation by host. Stained with toluidine blue/trypan blue (x 400 magnification).

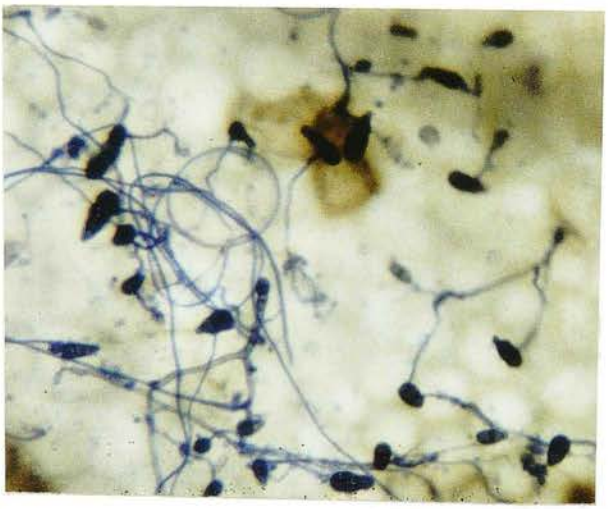


Fig. 5.2.

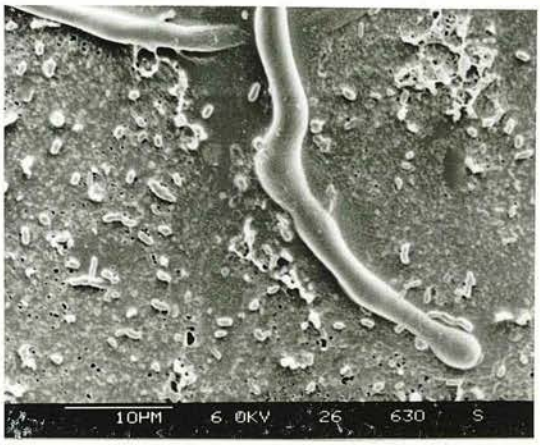


Fig. 5.3.

Fig. 5.4.

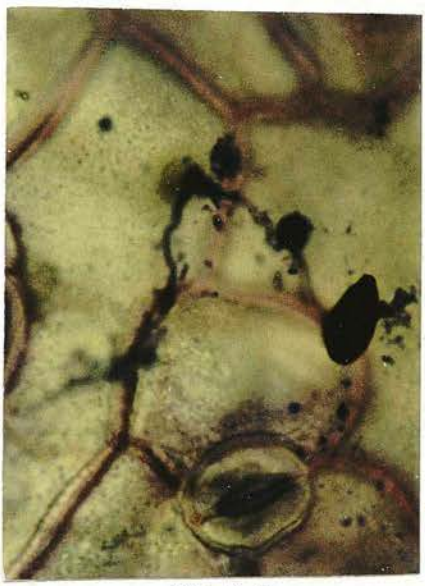


Fig. 5.5.

Fig. 5.6. Normal sub-cuticular hyphae of *Alternaria brassicicola* in leaf of wild type (C5), with fluorescent papillae formed by host at corners of epidermal cells. Stained with aniline blue/trypan blue (x 400 magnification).

Fig. 5.7. Proximal, distorted sub-cuticular hyphae of *Alternaria brassicicola* in leaves of 90GLO, originating from surface terminal vesicle. Stained with aniline blue/trypan blue (x 400 magnification).

Fig 5.8. Distal, distorted sub-cuticular hyphae of *Alternaria brassicicola* in leaves of 90GLO. Stained with aniline blue/trypan blue (x 400 magnification).

Fig. 5.6.

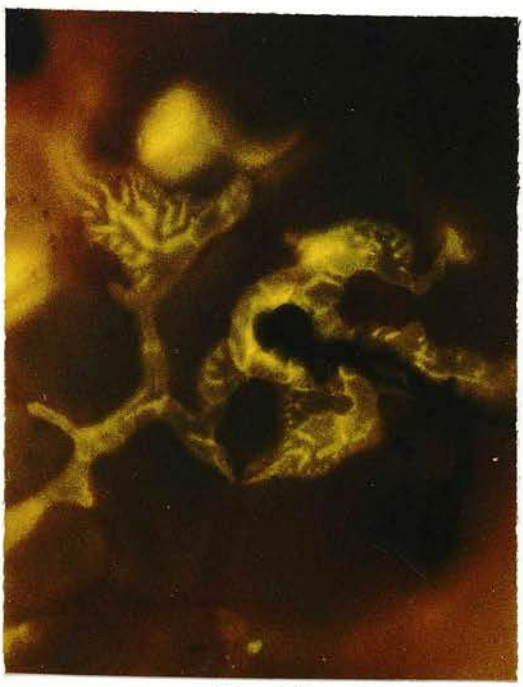


Fig. 5.7.

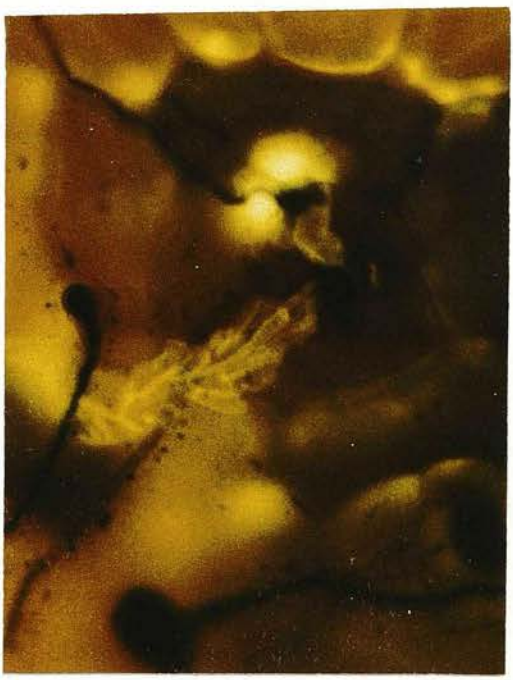


Fig.5.8.

Fig. 5.9. Sub-cuticular hyphae of *Alternaria brassicicola* in leaves of 90IN, following lines of middle lamella. Stained with aniline blue/trypan (x 400 magnification).

Fig. 5.10. Termination site of *Alternaria brassicicola* on 90GLO, showing terminal vesicle, sub-cuticular hyphae and necrosis of host epidermal cells. Stained with aniline blue/trypan blue (x 400 magnification).

Fig. 5.9.

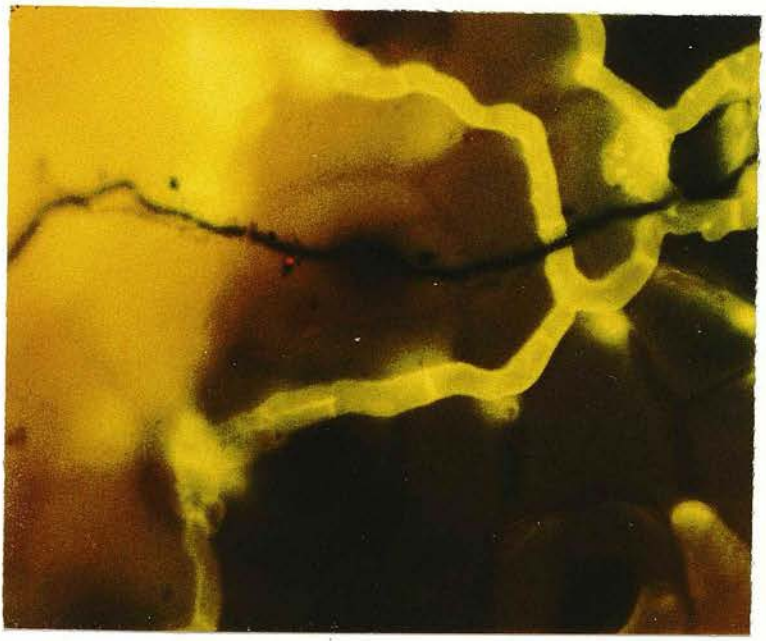


Fig. 5.10

strongly fluorescent walls or which had become completely fluorescent.

5.3.1. Leaf surface development and early penetration events of two **Alternaria** *species in relation to brassica plant and leaf position.*

Alternaria brassicicola.

Analyses of variance for the various effects are summarised in Appendix 5.2. In considering the effects of brassica type and leaf position on germination, in no instance were the differences in germination rate significant. Rates reached at least 87% and ranged up to 94% (Table 5.1a).

Most spores of *A. brassicicola* produced only one germ-tube but there was a significant effect of brassica plant on average germ-tube number and a significant interaction between brassica and leaf position. Average germ-tube number was lower on oilseed rape compared with other brassicas (Table 5.1b). No differences were found in numbers on different leaves of this brassica, but Brussels sprout apical leaves favoured germ-tube production. For swede the average number was higher on mid-leaves than on apical leaves. Differences, however, were slight.

The length of the primary germ-tube varied with brassica, leaf position and their interaction. It may be seen from Table 5.1c that germ-tubes grew most extensively on Brussels sprout apical leaves. Apical leaves of oilseed rape also supported extended growth.

Branching of the primary tube was infrequent but once again was noticeably higher on Brussels sprout apical leaves than on other leaves, hence producing a significant effect for the interaction between brassica and leaf position (Table 5.2a).

Generally the germ-tube diverged from the main axis once, except on

Table 5.1. Germination (%), germ-tube number and germ-tube length of Alternaria brassicicola in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Germination				
Swede	87.0	89.0	94.0	90.0
Oilseed Rape	86.9	94.0	91.0	90.6
Brussels sprout	92.2	91.0	92.2	91.8
Mean	88.7	91.3	92.4	90.8
(b) Germ-tube number				
Swede	1.1	1.3	1.2	1.2
Oilseed Rape	1.1	1.1	1.1	1.1
Brussels sprout	1.3	1.1	1.1	1.2
Mean	1.2	1.1	1.2	1.2
(c) Germ tube length (1 unit ≡ 45 μm)				
Swede	3.9	4.1	3.9	4.0
Oilseed Rape	6.2	3.2	4.2	4.5
Brussels sprout	10.6	3.8	4.6	6.3
Mean	6.9	4.5	6.3	5.0

SED:	(a)	(b)	(c)	d.f.
Brassica type	1.57	0.04	0.42	8
Leaf position	1.68	0.04	0.29	21
Brassica type x Leaf position	2.84	0.07	0.59	21
(at same level of brassica)	2.90	0.06	0.50	

Table 5.2. Branching, axis divergence and number of vesicles of primary germ-tube of Alternaria brassicicola in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Branching				
Swede	0.03	0.06	0.03	0.04
Oilseed Rape	0.00	0.03	0.07	0.03
Brussels sprout	0.51	0.00	0.03	0.19
Mean	0.20	0.03	0.04	0.09
(b) Axis divergence				
Swede	1.0	1.3	1.4	1.2
Oilseed Rape	1.0	1.3	1.1	1.1
Brussels sprout	2.1	0.9	1.0	1.4
Mean	1.4	1.2	1.2	1.2
(c) Vesicle number.				
Swede	0.9	1.0	1.0	1.0
Oilseed Rape	1.0	0.9	0.9	1.0
Brussels sprout	0.7	1.0	1.0	0.9
Mean	0.9	1.0	1.0	1.0

SED:	(a)	(b)	(c)	d.f.
Brassica type	0.02	0.17	0.02	8
Leaf position	0.02	0.12	0.03	21
Brassica type x Leaf position	0.04	0.24	0.05	21
(at same level of brasscia	0.04	0.21	0.05	

Brussels sprout apical leaves where two deviations were standard (Table 5.2b).

Vesicle production averaged almost one per primary germ-tube. There was a significant interaction between leaf position and brassica with apical leaves of Brussels sprout supporting slightly fewer vesicles than any other combination (Table 5.2c).

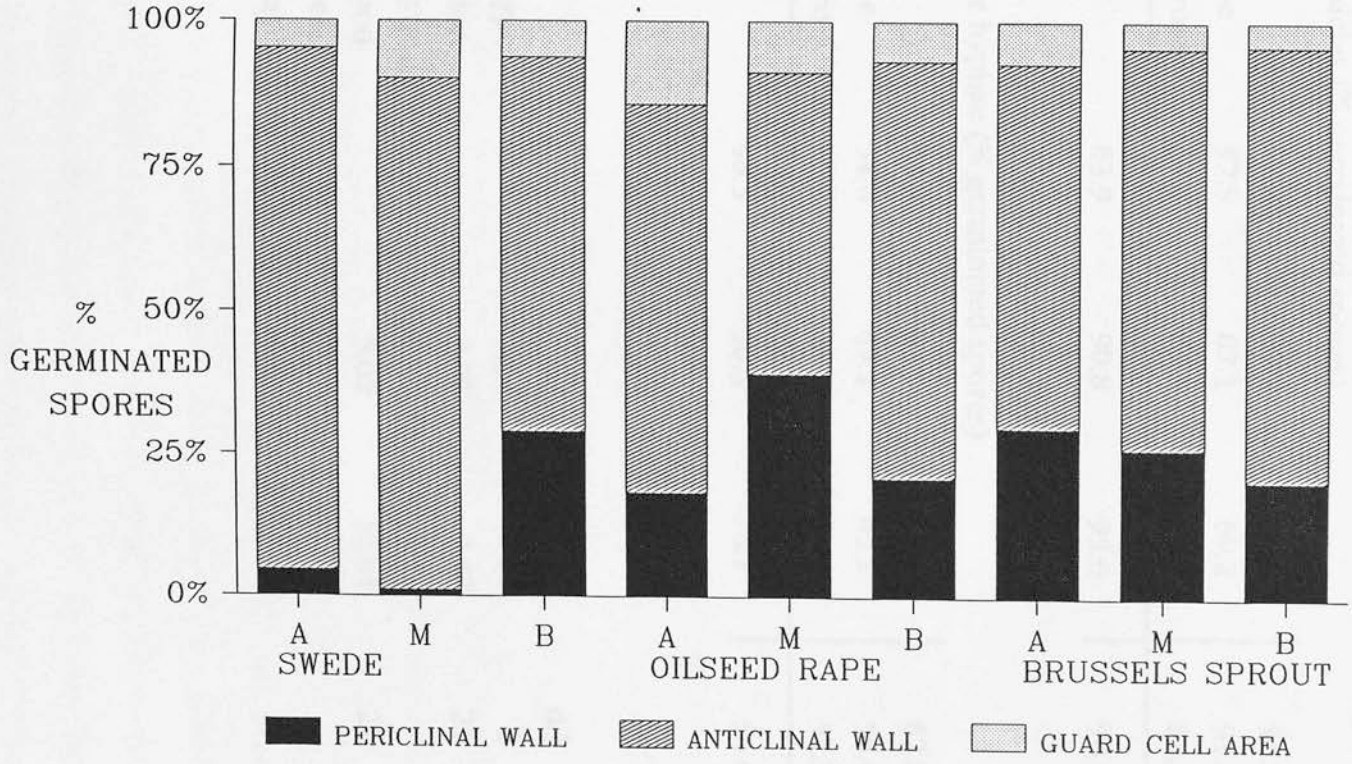
Analysis of the data showed that formation of terminal vesicles was influenced by brassica type, leaf position and their interaction. On average, apical leaves gave fewer terminal vesicles than other leaves but this was associated with particularly low numbers on Brussels sprout (Table 5.3a). This accounted for the low average score of Brussels sprout. Differences between brassicas on other leaves were small.

Germ-tubes could terminate at three designated sites on the leaf surface, the periclinal wall, anticlinal wall or guard cell area, which also included stomatal pores. The overall ratio for these sites was 2:7:1 (calculated from the grand means). Thus *A. brassicicola* preferentially favoured the anticlinal wall for germ-tube termination (Fig. 5.11).

In considering the individual analyses of variance for all three sites, anticlinal terminations were, on average, more frequent on apical leaves compared with middle and basal leaves. The reverse occurred with respect to periclinal wall termination. Differences between brassicas were not significant for any termination site, but there was an interaction between brassica and leaf position for periclinal and anticlinal walls. Leaf position did not seem to influence the site of termination of germ-tube growth on Brussels sprout, but on swede anticlinal wall termination was maximum on apical leaves and mid-

Fig. 5.11.

Sites of hyphal termination of *A. brassicicola* on apical (A), middle (M) and basal (B) leaves of different brassicas



SED= +/− PERICLINAL 3.96; ANTICLINAL 8.18; GUARD CELL 4.7

d.f.= 21

Table 5.3. Terminal vesicles (%) and sub-cuticular hyphae (%) produced by Alternaria brassicicola in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Terminal vesicles (% germinated spores)				
Swede	92.2	89.9	98.0	93.4
Oilseed Rape	97.9	87.1	89.2	91.4
Brussels sprout	61.7	95.5	99.7	85.6
Mean	83.9	90.8	95.6	90.1
(b) Sub-cuticular hyphae (% germinated spores)				
Swede	34.3	65.5	86.2	62.0
Oilseed Rape	54.0	49.1	63.9	55.6
Brussels sprout	57.3	36.3	63.1	52.2
Mean	48.5	50.3	70.1	56.6

SED:	(a)	(b)	d.f.
Brassica type	2.54	6.87	8
Leaf position	3.07	7.47	21
Brassica type x Leaf position	5.02	12.61	21
(at same level of brassica)	5.31	12.95	

leaves and periclinal wall least. With oilseed rape, anticlinal wall termination was less frequent on middle leaves where periclinal wall termination was more frequent. The incidence of germ-tube termination at guard cells was not significantly affected by either brassica or leaf position.

Brassica plant did not significantly influence the success or failure of a penetration, as evidenced by the frequency of sub-cuticular hyphae (Table 5.3b), although sub-cuticular hyphae were found slightly more often on swede than on other brassicas. Overall basal leaves allowed more sub-cuticular development than apical or mid-leaves, most notably on swede. Failed penetration tended to be more frequent in swede on apical leaves and in oilseed rape and Brussels sprout on middle leaves.

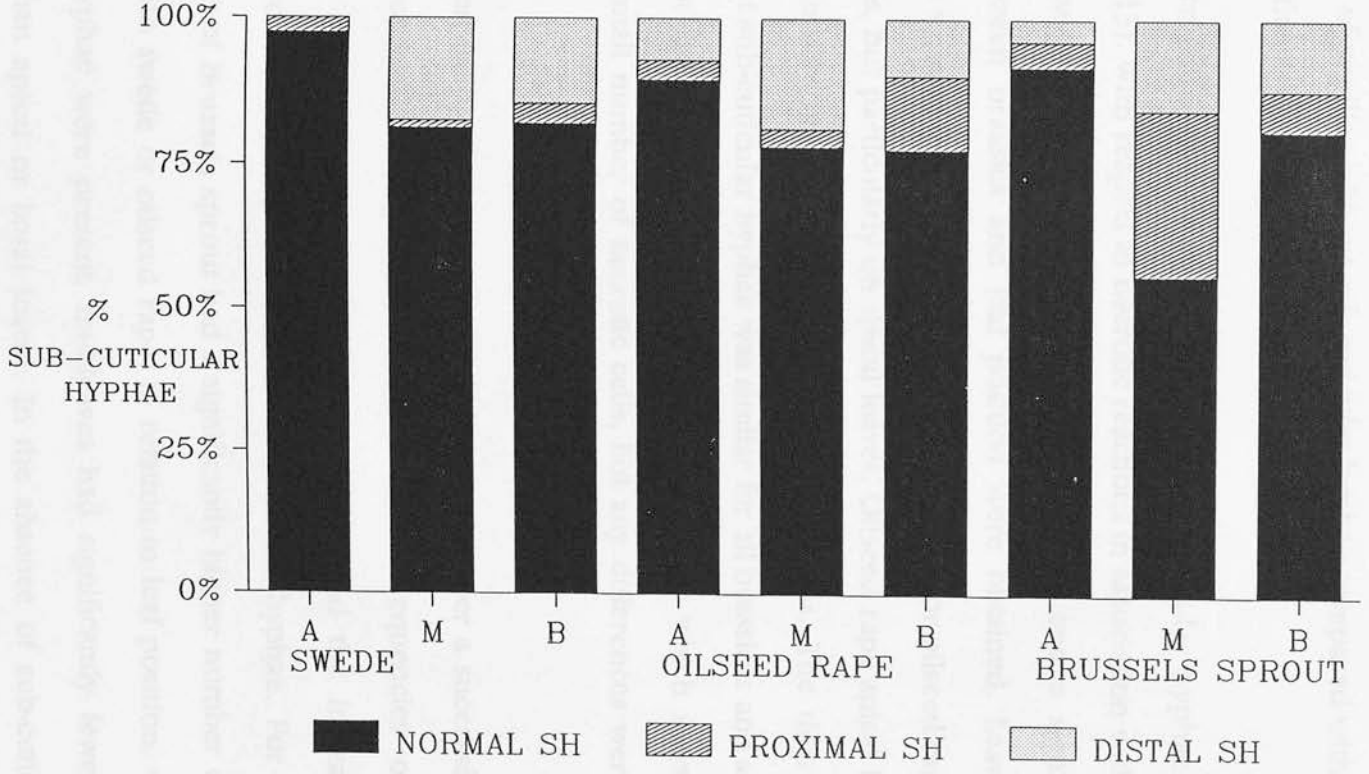
The majority of sub-cuticular hyphae were normal and only leaf position in general seemed to influence significantly differentiation into swollen structures (Fig 5.12). Distorted hyphal growth occurred mainly on mid- and basal leaves and was slightly more prevalent away from the site of penetration, although there was a fairly high frequency of swollen hyphae near to the site of penetration on the middle leaves of Brussels sprout.

Reactions of the hosts were either in the form of papillae, necrosis or absent and arose in presence or absence of sub-cuticular hyphae. The frequencies of all categories were analysed.

Overall papillae were formed in only about 15% of attempted penetrations. Swede leaves tended to form more papillae than oilseed rape and Brussels sprout. Analysis of the data showed a significant main effect for leaf position for formation of papillae associated with sub-cuticular hyphae, and there was a significant interaction between brassica and leaf position for

Fig. 5.12.

Types of sub-cuticular (SH) hyphae of *A. brassicicola* on apical (A), middle (M) and basal (B) leaves of different brassicas



SED= +/- NORMAL 10.76; PROXIMAL 7.80 ; DISTAL 6.53
d.f.= 21

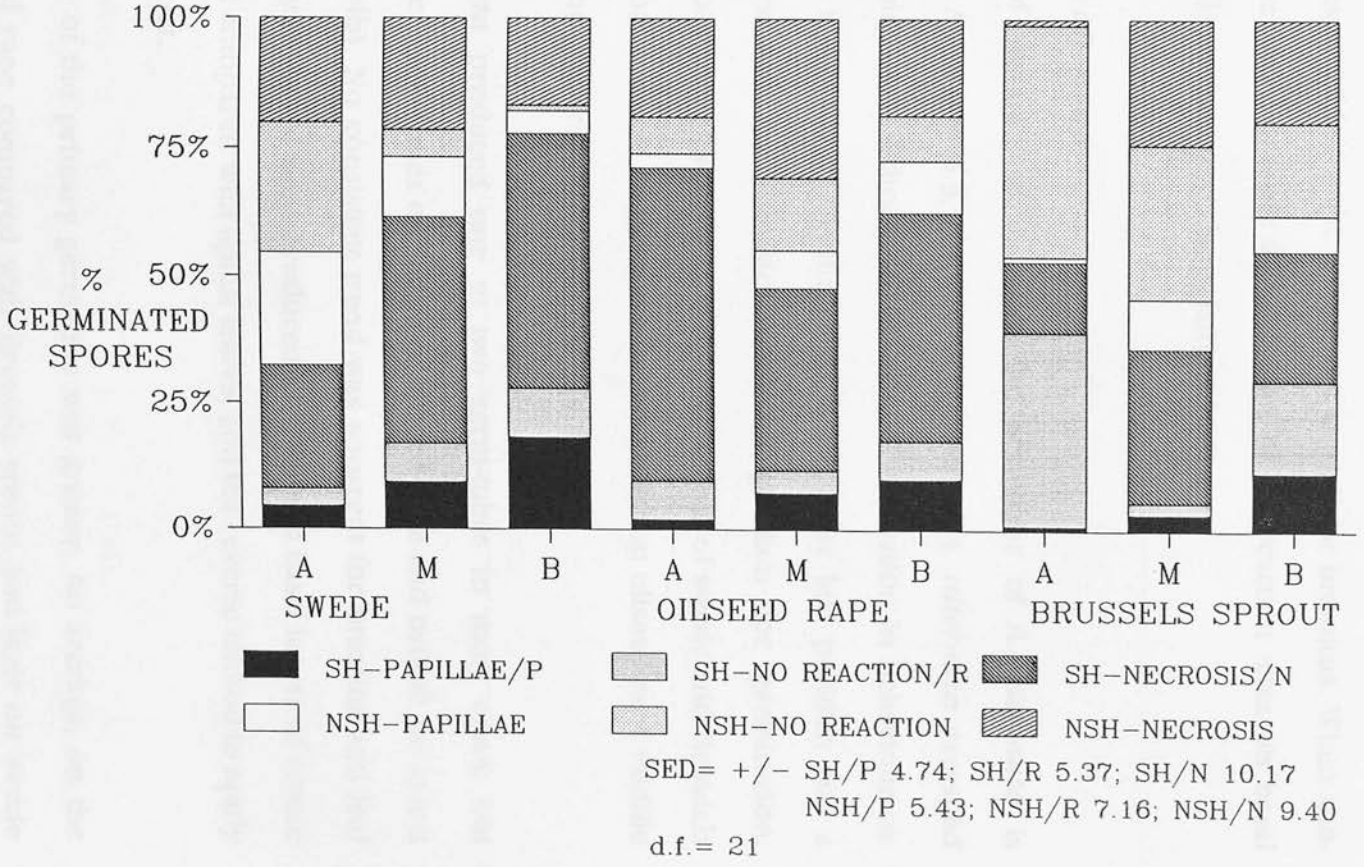
frequency of papillae without sub-cuticular hyphae (Fig. 5.13). If sub-cuticular hyphae were present, the numbers of papillae increased with leaf age *i.e.* at lower leaf positions. This was true not only overall but also for each individual brassica. No such general trends were evident for papillae formed without sub-cuticular hyphae but apical leaves of swede had a significantly higher number of papillae without sub-cuticular hyphae compared with other brassicas and other leaf positions.

Necrotic reactions were more frequent if sub-cuticular hyphae were present (Fig. 5.13). With respect to necrotic reactions in association with sub-cuticular hyphae, a significant main effect for brassica and a significant interaction between brassica and leaf position were obtained. Leaves of Brussels sprout showed less necrosis than leaves of swede or oilseed rape for all leaf positions, but particularly on apical leaves. Oilseed rape apical leaves had an exceptionally large number of necrotic reactions. The degree of necrosis without sub-cuticular hyphae was similar for all brassicas and all leaf positions except for the apical leaves of Brussels sprout which showed a comparatively small number of necrotic cells, but any differences were not significant.

On occasion the epidermal cells did not react to either a successful or unsuccessful penetration (Fig 5.13). Differences in frequencies of no reactions were significant for brassica, leaf position and the interaction between the two factors, with or without sub-cuticular hyphae. For both categories leaves of Brussels sprout had a significantly higher number of no reaction sites than swede or oilseed rape. In relation to leaf position, when sub-cuticular hyphae were present, mid-leaves had significantly fewer no reaction sites than apical or basal leaves. In the absence of sub-cuticular

Fig. 5.13.

Host cell reaction in presence (SH) or absence (NSH) of sub-cuticular hyphae of *Alternaria brassicicola* on apical (A), middle (M) and basal (B) of different brassicas



SED= +/- SH/P 4.74; SH/R 5.37; SH/N 10.17
NSH/P 5.43; NSH/R 7.16; NSH/N 9.40

d.f.= 21

hyphae numbers decreased with increasing leaf age, basal leaves having significantly fewer no reaction sites than apical or mid-leaves. In considering the interaction between brassica and leaf position, whether or not sub-cuticular hyphae were present, the apical leaves of Brussels sprout had a significantly higher number of no reaction sites than any other leaves, and this was probably responsible for the differences overall for brassicas. When sub-cuticular hyphae were present the numbers of no reaction sites on basal leaves of Brussels sprout were also relatively high.

Alternaria alternata.

Analysis of variance of the data for behaviour of *A. alternata* is summarised in Appendix 5.3. Germination rates of *A. alternata* averaged about 90% (Table 5.4a). Although there was little variation in behaviour on different leaves there was a significant main effect for leaf position and a significant interaction for brassica and leaf position for germination. Germination was significantly greater on apical leaves of swede and Brussels sprout than on basal leaves of these two brassicas but on oilseed rape middle leaves showed the higher germination rate (Table 5.4a).

A. alternata produced one to two germ-tubes in most cases, but significantly fewer germ-tubes on leaves of oilseed rape and overall on apical leaves (Table 5.4b). No consistent trend was apparent for brassica and leaf position. More germ-tubes were produced on mid- and basal leaves of swede and oilseed rape compared with apical leaves, and the reverse tended to apply for Brussels sprout.

The length of the primary germ-tube was greater, on average, on the leaves of oilseed rape compared with Brussels sprout and least on swede

Table 5.4. Germination (%), germ-tube number and germ-tube length of *Alternaria alternata* in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Germination				
Swede	97.0	89.0	90.0	92.0
Oilseed Rape	90.9	93.9	89.0	91.0
Brussels sprout	95.0	86.3	86.3	89.2
Mean	94.0	89.7	88.4	90.7
(b) Germ-tube number				
Swede	1.3	1.8	1.8	1.6
Oilseed Rape	1.2	1.4	1.5	1.4
Brussels sprout	1.7	1.5	1.4	1.5
Mean	1.4	1.6	1.6	1.5
(c) Germ tube length (1 unit ≡ 45 μm)				
Swede	2.6	1.8	3.7	2.7
Oilseed Rape	3.4	6.3	4.7	4.8
Brussels sprout	4.6	3.4	4.0	4.0
Mean	3.5	3.8	4.2	3.8

SED:	(a)	(b)	(c)	d.f.
Brassica type	1.41	0.09	0.42	8
Leaf position	1.22	0.07	0.31	21
Brassica type x Leaf position	2.23	0.13	0.60	21
(at same level of brassica)	2.12	0.12	0.53	

Table 5.5. Branching, axis divergence and number of vesicles of primary germ-tube of Alternaria alternata in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Branching				
Swede	0.06	0.08	0.16	0.10
Oilseed Rape	0.01	0.30	0.06	0.12
Brussels sprout	0.03	0.02	0.48	0.18
Mean	0.04	0.13	0.23	0.13
(b) Axis divergence				
Swede	0.8	1.1	0.7	0.9
Oilseed Rape	0.8	1.9	0.9	1.2
Brussels sprout	1.1	1.0	1.2	1.1
Mean	0.9	1.3	0.9	1.0
(c) Vesicle number.				
Swede	0.9	1.0	1.1	1.0
Oilseed Rape	0.9	1.0	0.8	0.9
Brussels sprout	0.7	0.7	0.5	0.6
Mean	0.8	0.9	0.8	0.8

SED:	(a)	(b)	(c)	d.f.
Brassica type	0.09	0.15	0.07	8
Leaf position	0.09	0.14	0.06	21
Brassica type x Leaf position	0.15	0.24	0.12	21
(at same level of brassica)	0.15	0.24	0.11	

(Table 5.4c). Basal leaves tended to support longer growth. However, leaf position effects differed with different brassicas, particularly on middle leaves where oilseed rape gave relatively long germ-tubes.

Branching rarely occurred on germ-tube hyphae of *A. alternata.* However, significant effects were obtained for the interaction between brassica and leaf position. This was due mainly to comparatively frequent branching occurring on the basal leaves of Brussels sprout and, in smaller part, to the branching on middle leaves of oilseed rape (Table 5.5a).

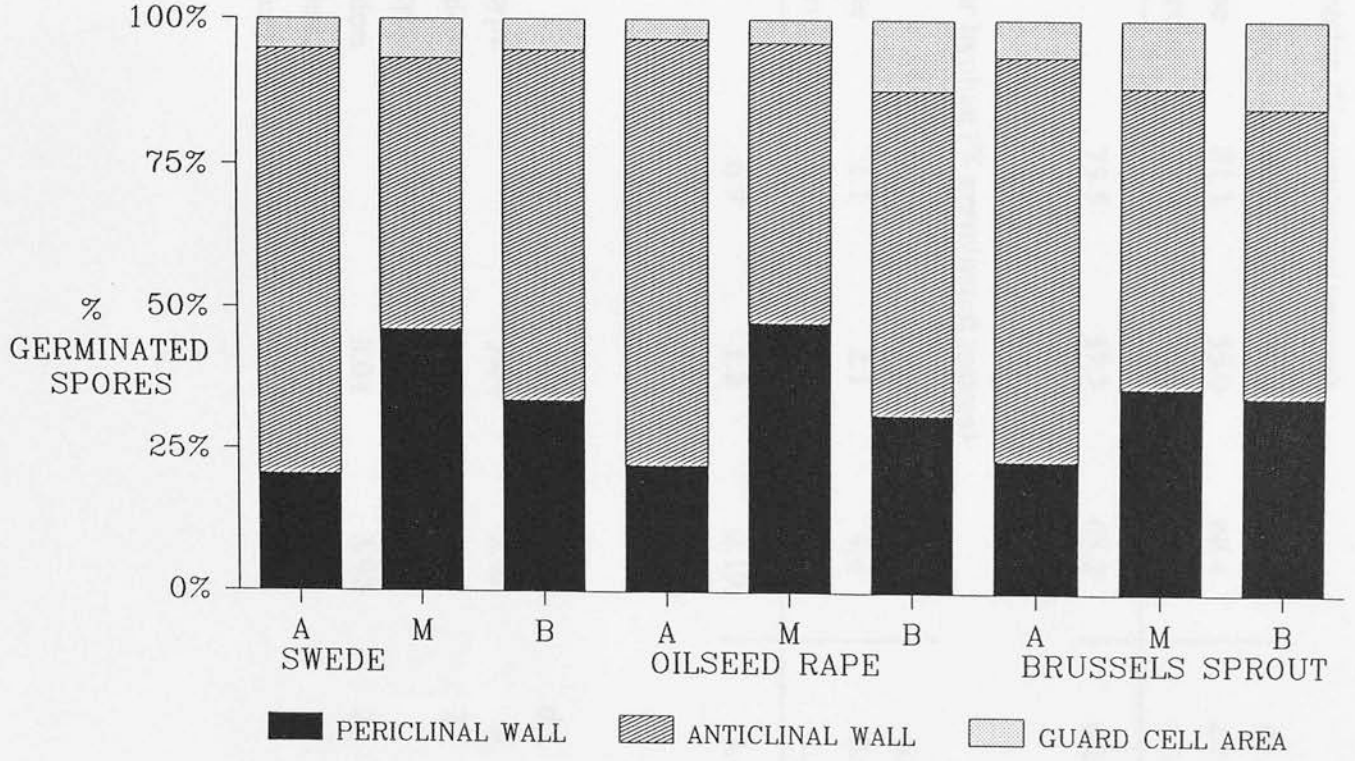
A significant main effect of leaf position and a significant interaction between brassica and leaf position were seen for axis divergence. Typically *A. alternata* produced one divergence except on mid-leaves of oilseed rape where an average of almost two was produced (Table 5.5b). Swede and oilseed rape showed similar trends in relation to leaf position: the mid-leaves caused the germ-tube to diverge from the main axis more often than on apical or mid-leaves. There was little difference in incidence of axis divergence for different leaves of Brussels sprout.

A significant effect of brassica type on vesicle numbers was found. Mostly one vesicle was produced in the primary germ-tube, except for Brussels sprout on which there were fewer germ-tubes which produced vesicles (Table 5.5c).

Alternaria alternata produced fewer terminal vesicles than *A. brassicicola* overall. The percentage of *A. alternata* germ-tubes ending in a vesicle was influenced by brassica and leaf position. On swede leaves there was the largest number of terminal vesicles, followed by oilseed rape then Brussels sprout leaves (Table 5.6a). Differences between all brassicas were

Fig. 5.14.

Sites of hyphal termination of *A. alternata* on apical (A), middle (M) and basal (B) leaves of different brassicas



SED= +/− PERICLINAL 8.00; ANTICLINAL 4.48; GUARD CELL 1.94
d.f.= 21

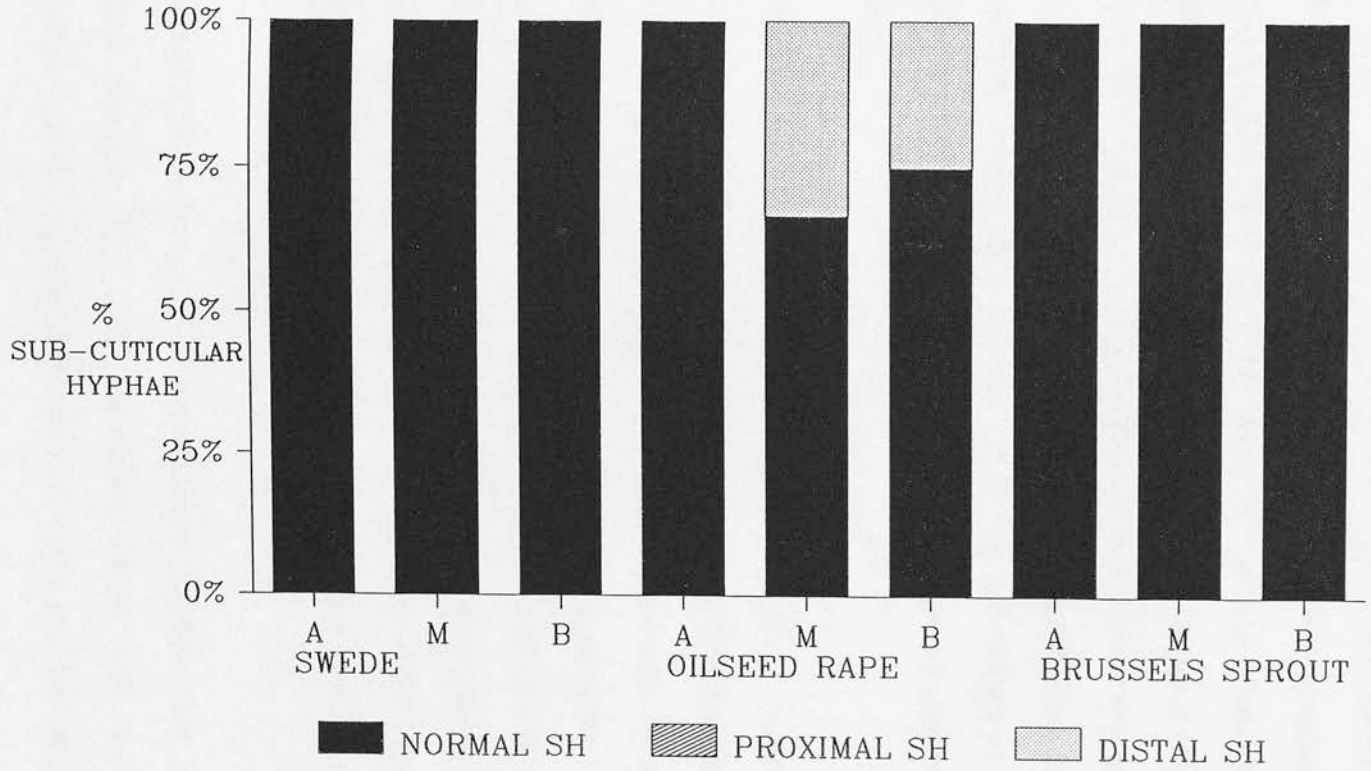
Table 5.6. Terminal vesicles (%) and sub-cuticular hyphae (%) produced by Alternaria alternata in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Terminal vesicles (% germinated spores)				
Swede	86.7	75.2	82.0	81.3
Oilseed Rape	81.1	53.0	68.4	67.5
Brussels sprout	58.7	45.0	39.2	47.6
Mean	75.5	57.7	63.2	65.5
(b) Sub-cuticular hyphae (% germinated spores)				
Swede	16.6	2.2	1.1	6.6
Oilseed Rape	1.1	2.1	4.4	2.6
Brussels sprout	3.1	0.8	0.6	1.0
Mean	6.9	1.2	2.19	3.4

SED:	(a)	(b)	d.f.
Brassica type	5.84	2.42	8
Leaf position	3.91	2.18	21
Brassica type x Leaf position	8.04	3.92	21
(at same level (of brassica)	6.77	3.78	

Fig. 5.15.

Types of sub-cuticular (SH) hyphae of *A. alternata* on apical (A), middle (M) and basal (B) leaves of different brassicas



SED= +/- NORMAL 26.59; PROXIMAL 0.0; DISTAL 2.71
d.f.= 21

significant. Numbers of terminal vesicles were significantly higher on apical leaves than on mid- or basal leaves.

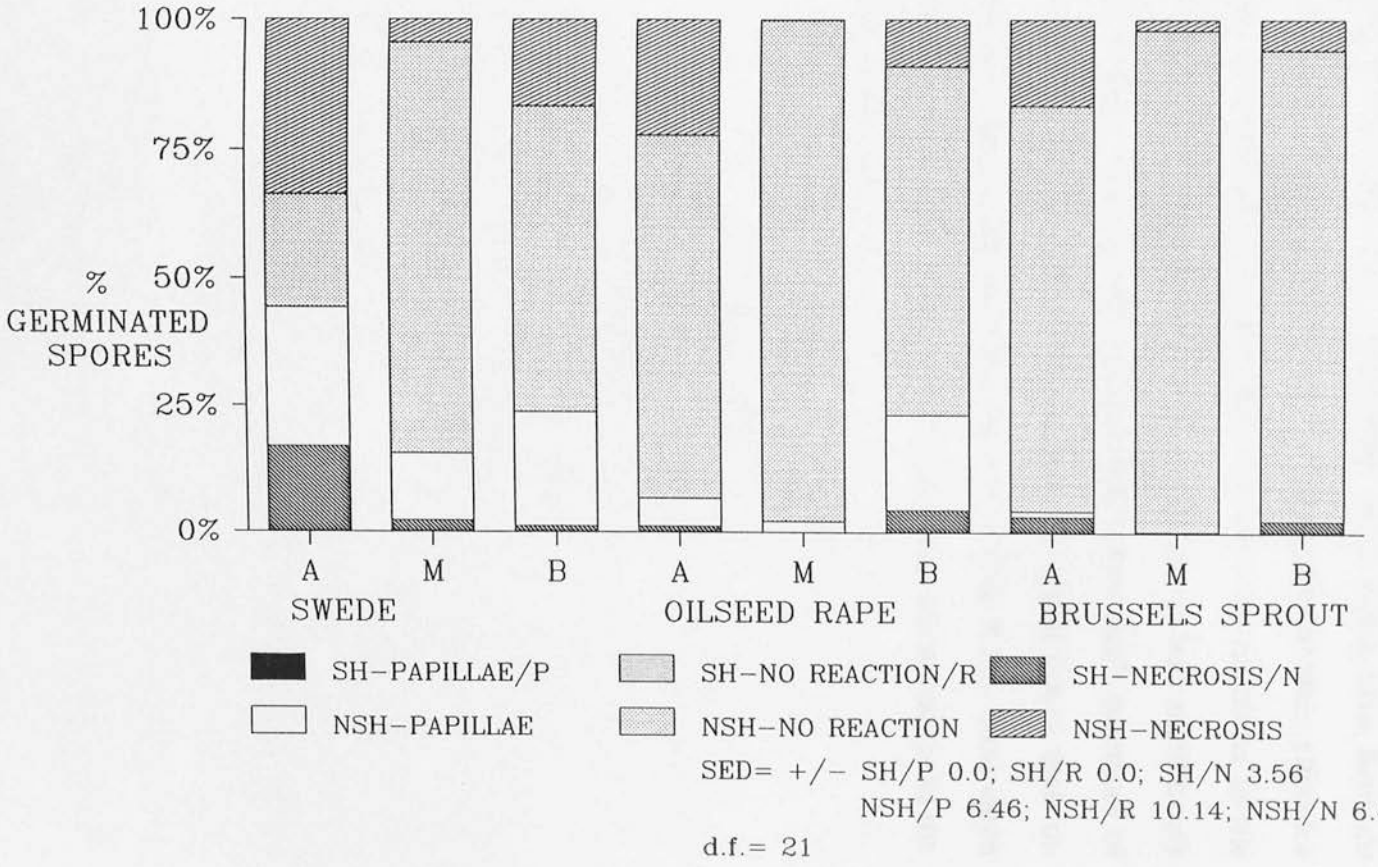
The overall ratio for hyphal growth termination sites was 3:6:1 for periclinal, anticlinal and guard cell walls, indicating that *A. alternata* showed a less marked response to anticlinal wall positions than *A. brassicicola* (Fig. 5.14). A significant main effect for leaf position only was obtained for frequency of terminal vesicles at periclinal and anticlinal wall sites, whereas no significant effects were seen for guard cells. At all leaf positions most germ-tubes ended in a vesicle at anticlinal wall sites but the proportion was greatest on apical leaves. The percentage of periclinal sites showing terminal vesicles was highest on middle leaves. Although not significant, percentage of guard cells sites with terminal vesicles was greatest on basal leaves, of oilseed rape and Brussels sprout.

Few attempted penetrations by *A. alternata* were successful. Nevertheless a significant main effect was obtained for leaf position and the interaction between brassica and leaf position for the presence of a sub-cuticular hyphae. It appeared swede leaves were significantly more susceptible than oilseed rape or Brussels sprout, but particularly when comparing the apical leaves (Table 5.6b). Almost all of successful penetrations resulted in normal sub-cuticular hyphae. Only the mid- and basal leaves of oilseed rape caused a small number of sub-cuticular hyphae to distort (Fig. 5.15).

Host epidermal cells responded to sub-cuticular hyphae only in the form of a necrotic reaction (Fig 5.16). Significant effects were seen for brassica, leaf position and the interaction between brassica and leaf position.

Fig. 5.16.

Host cell reaction in presence (SH) or absence (NSH) of sub-cuticular hyphae of *Alternaria alternata* on apical (A), middle (M) and basal (B) of different brassicas



SED= +/- SH/P 0.0; SH/R 0.0; SH/N 3.56
NSH/P 6.46; NSH/R 10.14; NSH/N 6.45

d.f.= 21

Significantly more necrotic sites were seen on leaves of swede, compared with oilseed rape and Brussels sprout but especially on the apical leaves.

In the absence of sub-cuticular hyphae some sites showed papillae formation, or necrosis. Overall, swede leaves were most active in forming papillae but oilseed rape was also significantly more active than Brussels sprout leaves, on which numbers were almost nil. A significant main effect for both brassica and leaf position was also seen for necrotic reactions in the absence of sub-cuticular hyphae. Once again swede leaves had significantly more necrotic sites than oilseed rape and Brussels sprout and numbers of necrotic sites were seen to be significantly higher on apical leaves than on other leaves, very few sites being seen on mid-leaves (Fig. 5.16). Most often there was no reaction of the host to apparent failed penetrations except in the case of apical leaves of swede.

5.3.2. Leaf surface development and early penetration events of two **Alternaria** *species in relation to* **gemmifera** *mutant line.*

Alternaria brassicicola

The analyses of variance of the data for the various assessments of fungal development and host response are given in Appendix 5.4.

Differences in germination rate of *Alternaria brassicicola* spores were not significant for different mutant lines, with percentages ranging from 88 to 98% (Table 5.7). The number of germ-tubes produced was, however, significantly higher on the intermediate phenotype of line 90 than on other plants. Overall 50% more germ-tubes was produced on this surface compared with the other phenotypes (Table 5.7). Germ-tube length was slightly affected by the leaf surface of different mutant lines (Table 5.7), the intermediate type of line 90 giving the longest germ-tube and the wild type the shortest.

Branching once more was infrequent but was significantly higher on the intermediate phenotype of line 90 than on any other mutant. Apart from this mutant, incidence of branching was rare (Table 5.7).

Despite a significant effect for mutant line in relation to axis divergence, it could not be related to degree of waxiness. Generally the germ-tube made usually one deviation except on the intermediate phenotypes of lines 90 and 229 and the glossy phenotype of line 229 when two were quite often produced (Table 5.7). There was no obvious effect of leaf surface phenotype on number of vesicles produced per germ-tube and although very occasionally more were produced on the waxy and intermediate phenotype of line 229, all values approached one (Table 5.8).

Table 5.7. Development of Alternaria brassicicola on leaves of gemmifera mutants.

MUTANT LINE	Germination (%)	Germ-tube number	Germ-tube length *	Branching	Axis divergence
C5	95.2	1.1	4.3	0.07	1.4
90WAX	88.0	0.9	4.6	0.06	1.1
90INT	90.4	1.5	5.4	0.25	1.6
90GLO	96.8	1.1	4.8	0.11	0.9
99GLO	94.4	1.1	5.2	0.07	0.9
229WAX	90.9	1.1	4.1	0.05	0.9
229INT	96.0	1.1	4.7	0.10	1.7
229GLO	98.4	1.1	4.4	0.05	1.8
SED = +/- (d.f. = 27)	2.86	0.13	0.43	0.05	0.23

*- 1 unit ≡ 45 μm

A. brassicicola formed a terminal vesicle from at least 90% of germ-tubes on all mutants except the waxy phenotype of line 90 (Table 5.8). Numbers produced on this surface were significantly lower than on any other surface, while the intermediate phenotype of this line also supported a significantly lower number of terminal vesicles than several other surfaces. No consistent relationship between numbers of terminal vesicles and degree of waxiness was found.

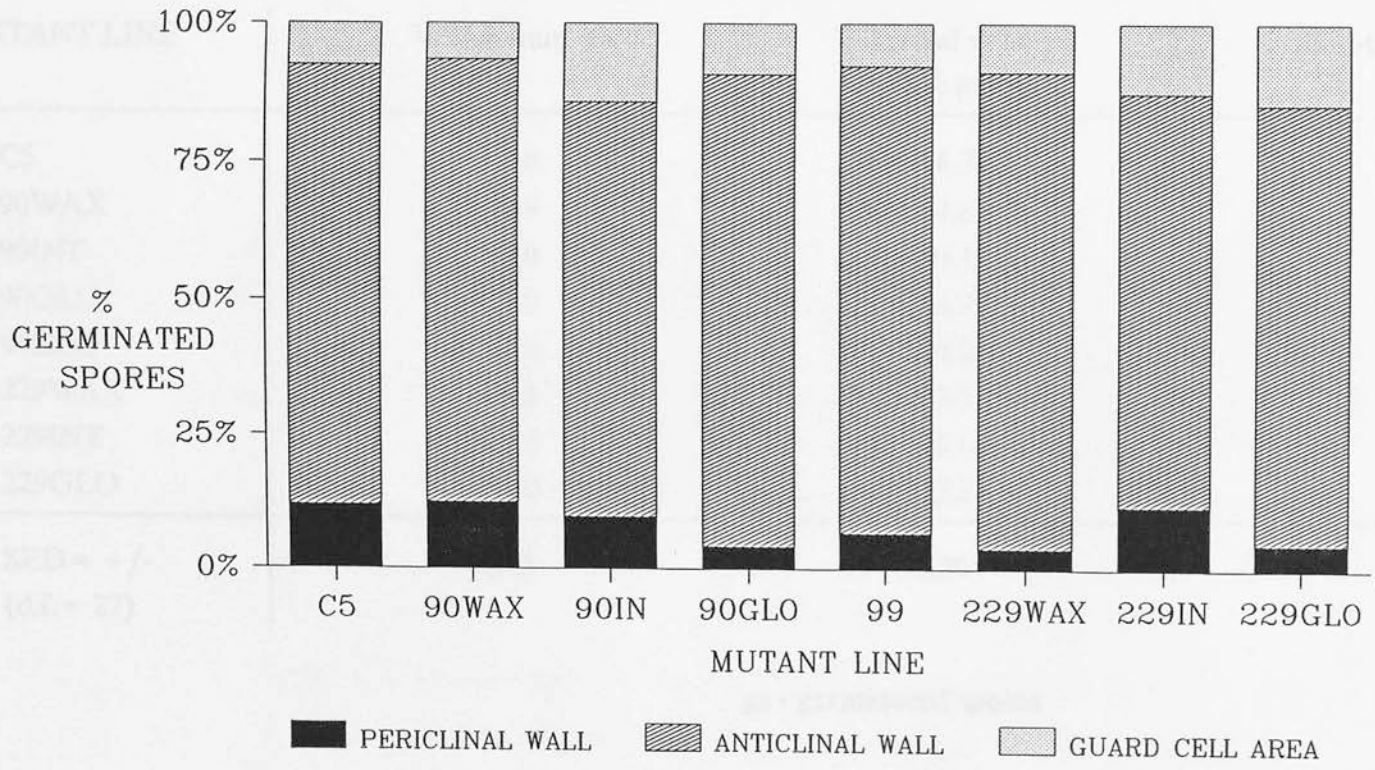
Surface type did not influence where germ-tubes terminated, no significant differences being obtained for periclinal, anticlinal or guard cell hyphal termination. However the overall ratio, calculated as 1:8:1 confirmed the preferential selection of anticlinal wall for termination of *A. brassicicola* germ-tubes found in the previous experiment. (Fig. 5.17).

The percentage of penetration sites giving rise to sub-cuticular hyphae varied significantly with mutant line, waxy surfaces having fewest sub-cuticular hyphae and glossy surfaces the most (Table 5.8). The majority of sub-cuticular hyphae, overall, were normal but differences in proportions of normal sub-cuticular hyphae among different mutant lines were significant (Fig 5.18). Thus, in mutant lines 90 and 229 the percentages of normal hyphae increased with increasing waxiness. However, the wild type, C5, with a waxy surface, gave a relatively low percentage of normal hyphae and the 99GLO, with a glossy surface, gave a high percentage. The variation in frequency of abnormal sub-cuticular hyphae was significant only with respect to proximal positions, glossy phenotypes of lines 90 and 229 showing the most, along with the wild type, and line 99GLO and 229WAX showing the least.

Attempted penetrations were seen to induce host cell reactions whether

Fig. 5.17.

Sites of hyphal termination of *Alternaria brassicicola* in relation to *gemmifera* mutant line



SED= +/− PERICLINAL 3.46; ANTICLINAL 4.66; GUARD CELL 3.46

d.f.= 27

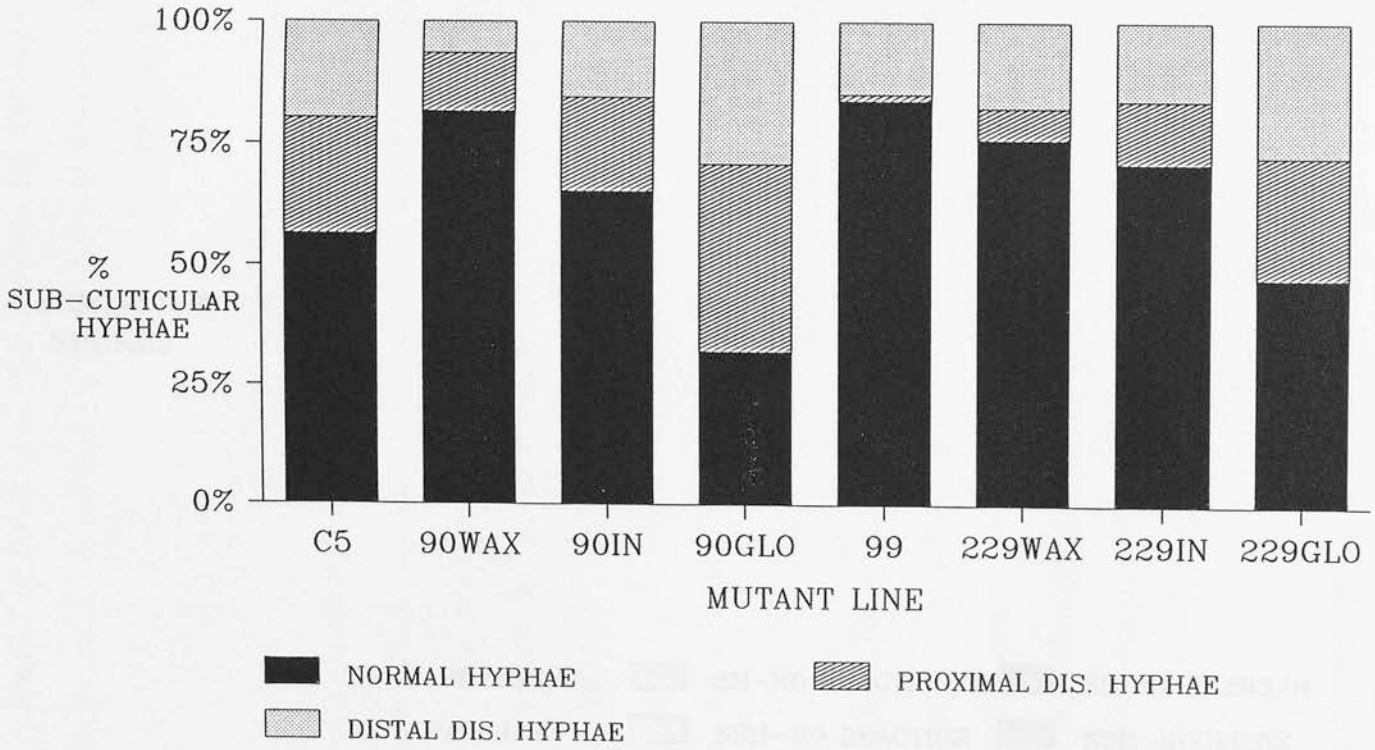
Table 5.8. Vesicle number, terminal vesicles and sub-cuticular hyphae produced by Alternaria brassicicola on leaves of gemmifera mutants.

MUTANT LINE	Vesicle number	Terminal vesicles (% gs)	Sub-cuticular hyphae (% gs)
C5	1.0	96.7	45.4
90WAX	0.9	82.8	33.5
90INT	1.0	90.1	60.1
90GLO	1.0	96.7	61.9
99GLO	1.0	94.0	63.6
229WAX	1.1	93.5	52.5
229INT	1.1	96.6	60.7
229GLO	1.0	93.5	72.3
SED = +/- (d.f. = 27)	0.05	2.30	5.99

gs - germinated spores

Fig. 5.18.

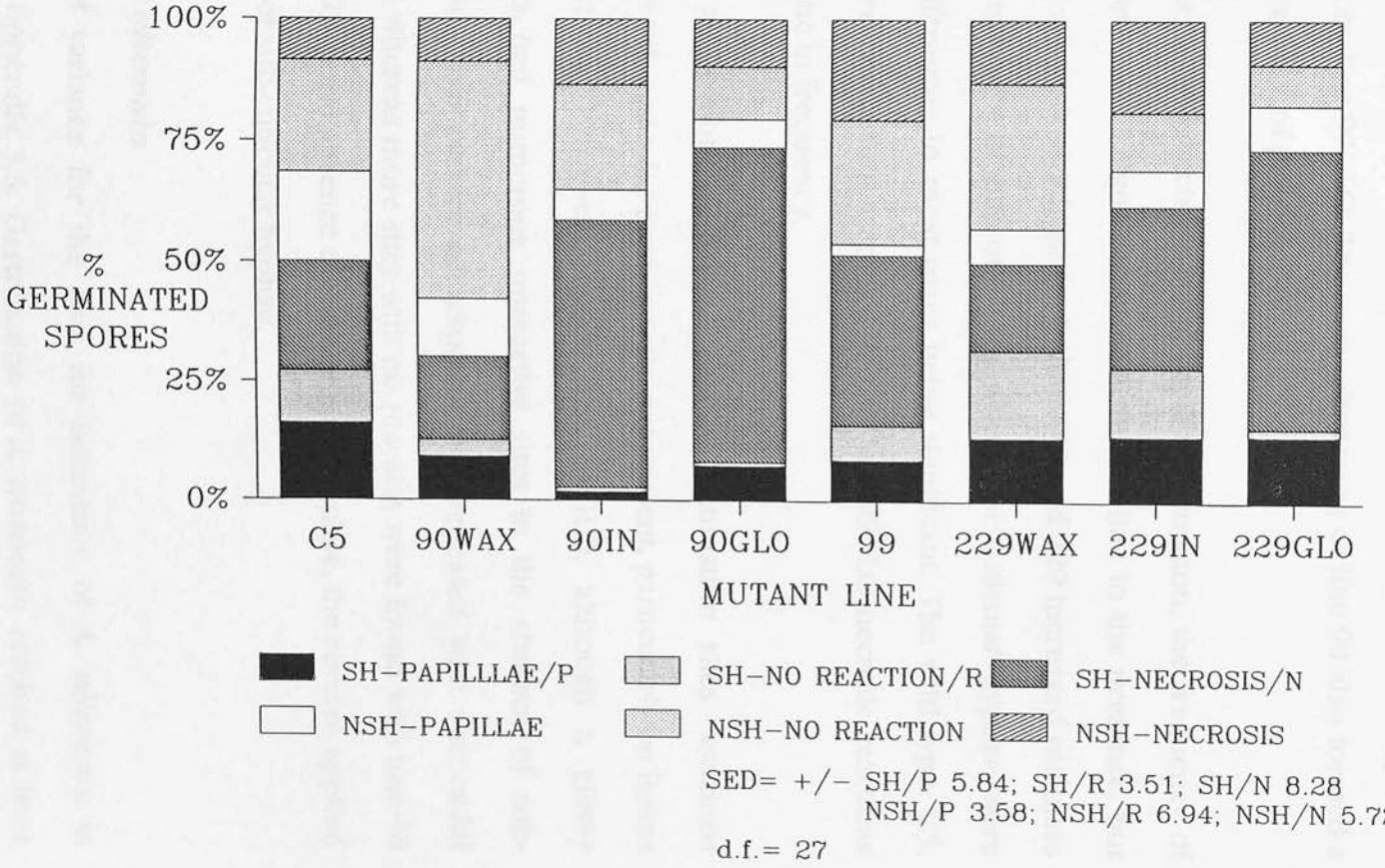
Sub-cuticular hyphal types of *Alternaria brassicicola* in relation to *gemmifera* mutant line



SED= +/- NORMAL 9.26; PROXIMAL 7.72; DISTAL 7.76
d.f.= 27

Fig. 5.19.

Host cell reactions in presence (SH) or absence (NSH) of *A. brassicicola* sub-cuticular hyphae on *gemmifera* mutants



or not penetrations were successful (Fig 5.19). Where sub-cuticular hyphae were produced leaf surface characteristics had no significant effect on papillae formation although the frequency of this category was very low in line 90IN. However, in the absence of sub-cuticular hyphae there were significant differences, papillae being produced most frequently by the wild type and least frequently by line 99GLO. The waxy phenotype of line 90 also formed a relatively high number of papillae.

In contrast to the effects seen for papillae formation, the frequency of necrotic reactions varied significantly with mutant line in the presence, but not absence, of sub-cuticular hyphae. In lines 90 and 229 increased waxiness gave reduced numbers of necrotic sites where sub-cuticular hyphae were present, the differences in most cases being significant. The wild type, C5, tended to give relatively little necrosis and in line 99GLO necrotic reactions were intermediate in frequency.

A larger proportion of hyphal growth termination sites remained unreactive when sub-cuticular hyphae were not present, particularly on leaves with waxy surfaces. However, mutant line 99GLO, although a glossy phenotype, also had numerous unreactive sites in the absence of sub-cuticular hyphae. This pattern of response was repeated with successful penetration, but whereas more sites with no reaction were found with line 90 than with line 229 in the absence of sub-cuticular hyphae, the reverse applied in the presence of sub-cuticular hyphae.

Alternaria alternata

Analysis of variance for the data for behaviour of *A. alternata* is summarised in Appendix 5.5. Germination of *A. alternata* reached at least

Table 5.9. Development of Alternaria alternata on leaves of gemmifera mutants.

MUTANT LINE	Germination (%)	Germ-tube number	Germ-tube length *	Branching	Axis divergence
C5	98.0	1.6	8.5	0.2	2.2
90WAX	99.0	1.6	5.6	0.4	2.0
90INT	98.0	1.7	8.1	0.2	1.6
90GLO	98.0	1.7	6.2	0.2	1.6
99GLO	98.8	1.2	4.6	0.0	1.1
229WAX	95.1	1.5	6.4	0.2	1.9
229INT	98.0	1.7	6.1	0.1	1.6
229GLO	97.0	1.5	7.8	0.1	2.0
SED = +/- (d.f. = 26)	1.69	0.09	0.79	0.11	0.25

*- 1 unit ≡ 45 μm

95% and any differences on different lines were not significant (Table 5.9). The numbers of germ-tubes produced per spore, on the other hand, varied significantly from an average of 1.7 on leaves of the glossy and intermediate phenotypes of mutant line 90 and the intermediate phenotype of line 229 to around one on line 99GLO (Table 5.9). Germ-tube length also varied significantly, the longest germ-tube being found on the wild type and the shortest on 99GLO. There were no obvious relationships between germ-tube length and leaf surface characteristics in parental lines (Table 5.9)

Branching occurred only very occasionally or not at all and was not significantly affected by mutant line (Table 5.9). The occurrence of axis divergence varied slightly on different lines being most frequent on the wild type and least frequent on 99GLO (Table 5.9).

Vesicle numbers (Table 5.10) were relatively high on the waxy phenotype of line 90 and tended in general to be most frequent on waxy surfaces.

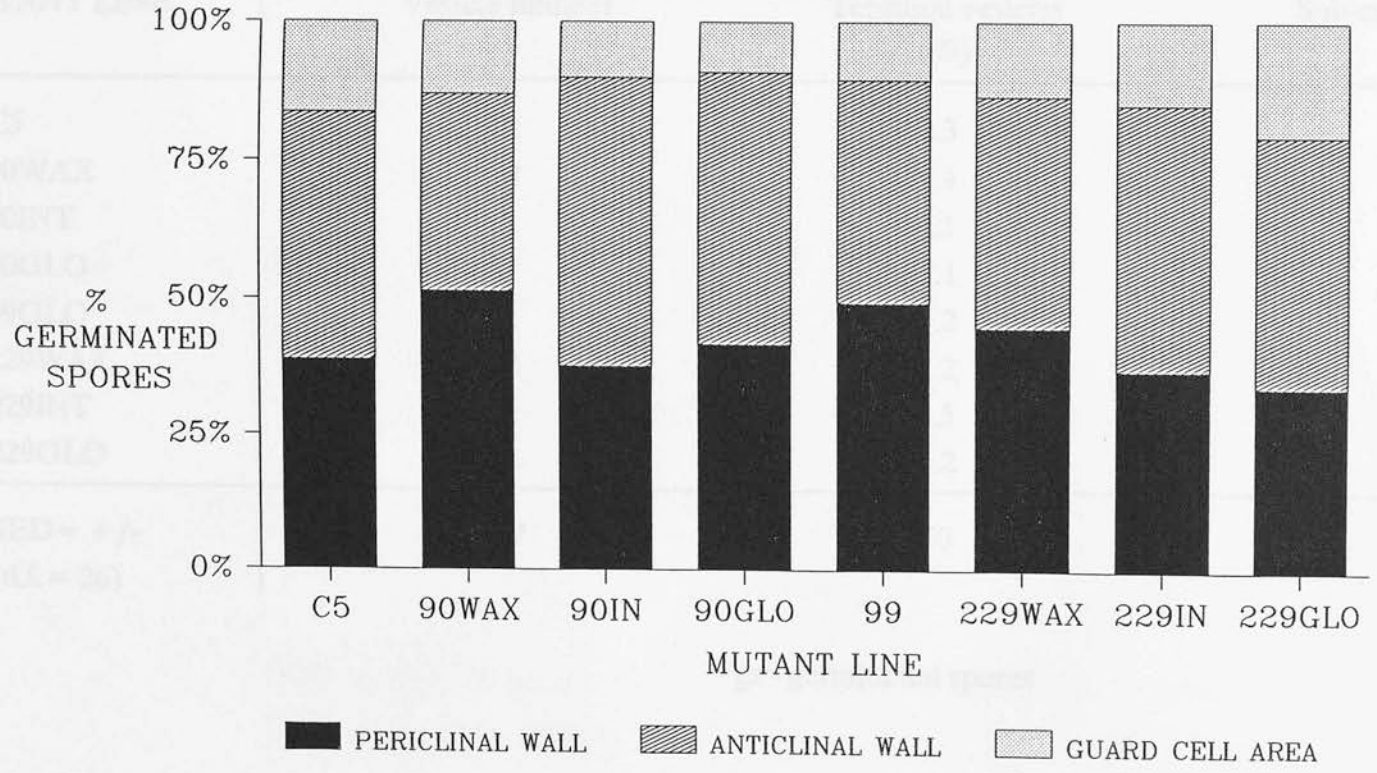
The frequency of terminal vesicles was dependant on mutant line and appeared to be partly related to the degree of surface waxiness (Table 5.10). The glossy surfaces of line 90 favoured terminal vesicles compared with the waxy surface, and the highest numbers occurred on 99GLO. Differences with parental line 229 were less marked than those in line 90, with respect to the effects of leaf surface.

The ratio of termination sites was 4:5:1 for periclinal, anticlinal and guard cell walls. Selection of termination site was not influenced by mutant line (Fig. 5.20).

Successful penetrations were infrequent but were seen to be significantly

Fig. 5.20.

Sites of hyphal termination of *Alternaria alternata* in relation to *gemmifera* mutant line



SED= +/− PERICLINAL 6.86; ANTICLINAL 7.75; GUARD CELL 4.10
d.f.= 26

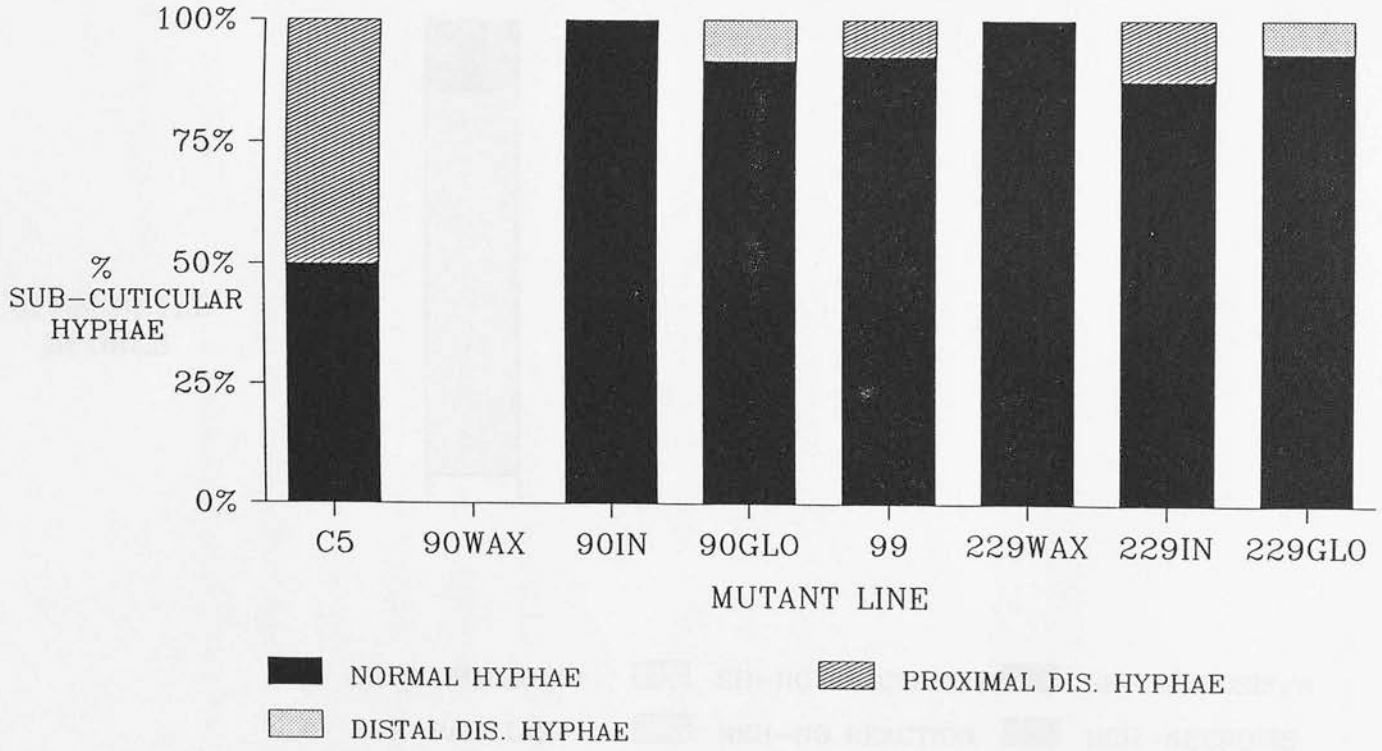
Table 5.10. Vesicle number, terminal vesicle and sub-cuitcular hyphae produced by Alternaria alternata on leaves of gemmifera mutants.

MUTANT LINE	Vesicle number	Terminal vesicles (%)	Sub-cuticular hyphae (%)
C5	1.2	50.3	4.1
90WAX	1.8	45.4	0.0
90INT	1.1	52.1	1.1
90GLO	1.1	65.1	13.0
99GLO	1.1	84.2	36.8
229WAX	1.3	61.2	4.5
229INT	1.1	58.5	6.7
229GLO	0.8	64.2	14.7
SED= +/- (d.f.= 26)	0.17	9.50	9.53

gs - germinated spores

Fig. 5.21.

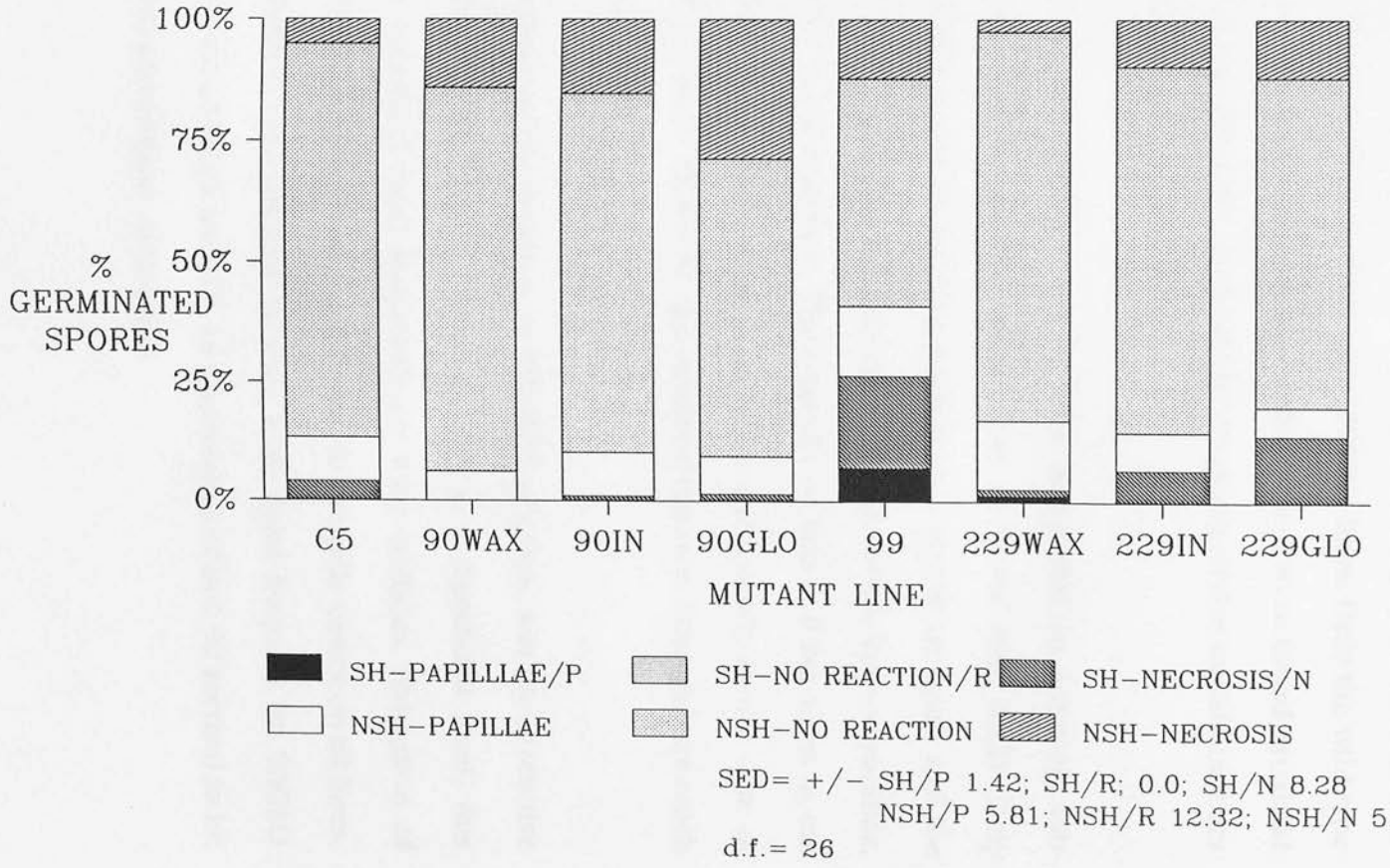
Sub-cuticular hyphal types of *Alternaria alternata* in relation to *gemmifera* mutant line



SED= +/- NORMAL 29.89; PROXIMAL 12.05; DISTAL 2.19
d.f.= 26

Fig. 5.22.

Host cell reactions in presence (SH) or absence (NSH) of *A. alternata* sub-cuticicular hyphae on *gemmifera* mutants



dependent on mutant line (Table 5.10). Numbers of sub-cuticular hyphae were greatest on glossy mutants, notably on line 99GLO, whereas the waxy phenotype of line 90 did not appear to allow formation of sub-cuticular hyphae. The type of sub-cuticular hyphae was not influenced by mutant line but as a rule sub-cuticular hyphae were not distorted (Fig 5.21). No distorted hyphae were found with 90WAX, 90IN and 229WAX lines. Only the wild type had a sizable proportion of differentiated hyphae which were found proximal to the supposed penetration site. It should be emphasised that actual numbers were generally very low.

Where penetrating hyphae which were successful in forming sub-cuticular structures, either papillae or necrosis occurred (Fig. 5.22). Only mutant line 99GLO reacted by forming papillae at a significant level, and the waxy phenotype of line 229 was the only other mutant which formed papillae, in this case in very small numbers. The majority of host cell reactions in the presence of sub-cuticular hyphae were necrotic particularly in the case of 99GLO and 229GLO lines. However, the variation between lines did not reach a significant level.

The most common observation, in 50 - 80% of cases, was an unreactive site without sub-cuticular hypha. Although not at a significant level, this observation was recorded most frequently on waxy surfaces. Formation of papillae without sub-cuticular hyphae occurred to a similar extent on all lines. Necrotic sites without sub-cuticular hyphae were most frequent on 90GLO and least frequent on 229WAX and C5; all phenotypes of line 90 seemed to be more reactive than phenotypes of line 229.

Erysiphe cruciferarum

The development of *Erysiphe cruciferarum* following 36 hours incubation is illustrated in Figs 5.23 to 5.28. Germination of *E. cruciferarum* conidia was by short germ-tubes ending in a large, lobed appressorium (Fig. 5.23) over one of the three designated sites previously described for *Alternaria.* From this, penetration of the surface took place.

If penetration was successful, an epidermal cell was invaded and a haustorium was established (Figs 5.24, 5.25 and 5.26). The epidermal cells invariably reacted to penetrating hyphae by forming papillae localised at the site of penetration (Figs 5.24, 5.25 and 5.26).

When association with the host was complete, further surface development occurred. Initially a hypha extended from the appressorium, from which further penetrations were attempted (Fig. 5.27). Subsequently germ-tubes were formed from each corner of the conidium, from below the appressorium or again from the appressorium itself (Figs 5.26 and 5.28 and 5.29). After a period of proliferation on the surface, all germ-tubes could branch and give rise to conidial initials (Fig. 5.30).

Fig. 5.23. SEM of conidium with appressorium of *Erysiphe cruciferarum* on leaf surface of 99GLO.

Fig. 5.24. Bright field micrograph of haustorium within epidermal cell of swede mid-leaf (x 1000 magnification).

Fig. 5.25 Fluorescence micrograph of haustorium within swede mid-leaf showing host wall reaction. Stained with aniline blue (x 1000 magnification).

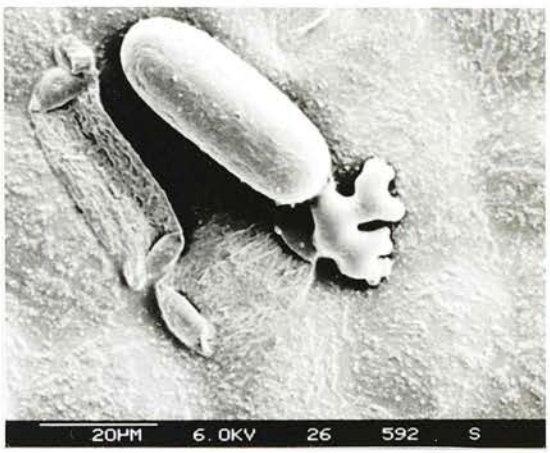


Fig. 5.23.

Fig. 5.24.

Fig.5.25.

Fig. 5.26. Development of *Erysiphe cruciferarum* on 90WAX leaf, showing secondary structures, haustorium within cells and host reaction. Stained with aniline blue/trypan blue (x 100 magnification).

Fig. 5.27. Development of secondary structures of *Erysiphe cruciferarum* on 90WAX leaf. Stained with trypan blue (x 400 magnification).

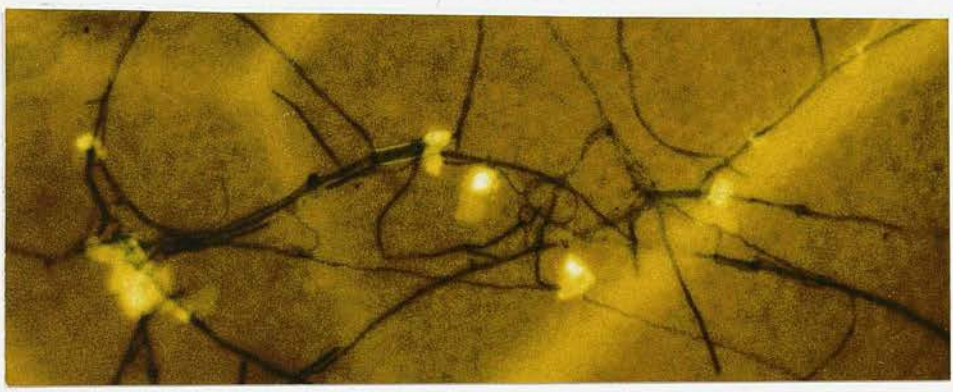


Fig. 5.26.

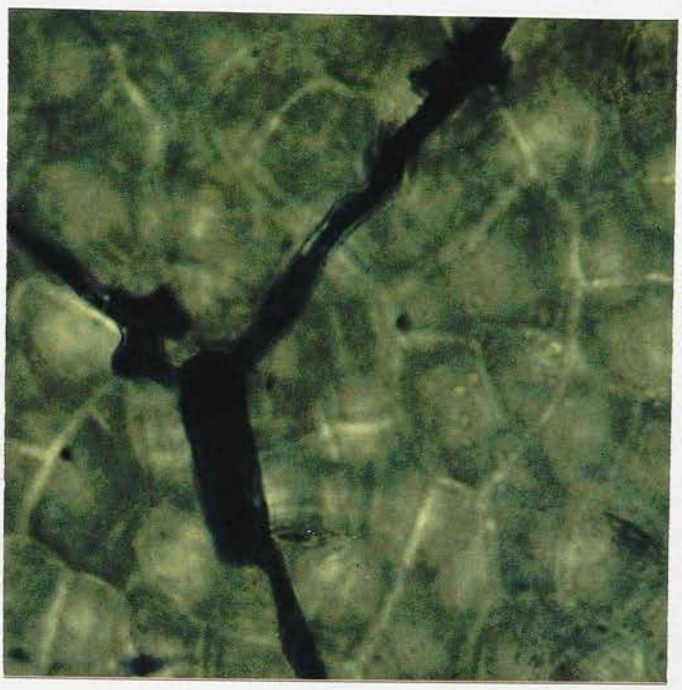


Fig. 5.27.

Fig. 5.28. SEM of *Erysiphe cruciferarum* development on 90IN leaf, showing secondary structures.

Fig. 5.29. SEM of *Erysiphe cruciferarum* development on 90WAX, showing secondary structures.

Fig 5.30. Development of *Erysiphe cruciferarum* on basal leaf of swede, showing secondary structures and conidial initials. Stained with aniline blue (x 100 magnification).

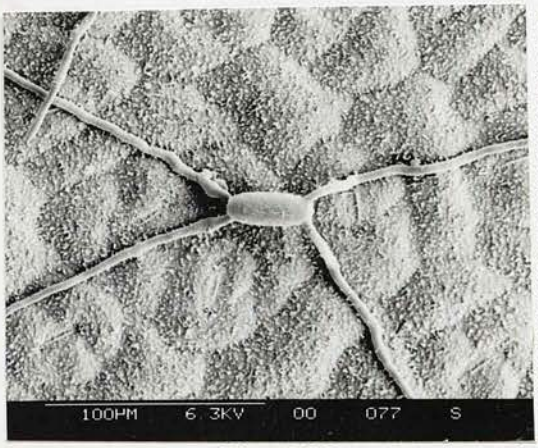


Fig. 5.28.

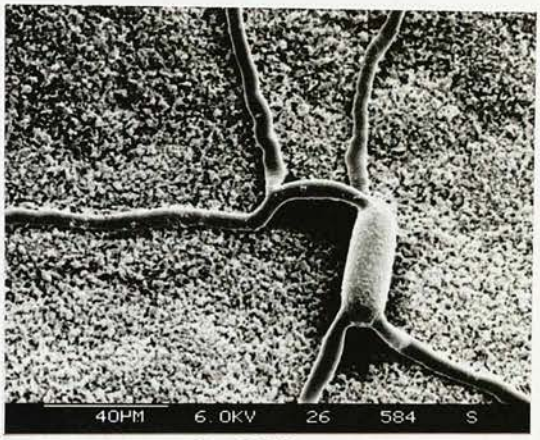


Fig 5.29.

Fig. 5.30.

5.3.3. Leaf surface behaviour and early penetration events of **Erysiphe cruciferarum** *in relation to brassica plant and leaf position.*

The analysis of variance of all the data for this experiment is given in Appendix 5.6. At least 50% of conidia germinated, regardless of brassica or leaf position (Table 5.11a). The length of the primary germ-tubes varied from 0.4-0.6 units, and was slightly greater on apical leaves (Table 5.11b). About 97% of germinated conidia produced an appressorium irrespective of brassica or leaf position (Table 5.11c). The size of these appressoria varied slightly, but not significantly with brassica and leaf position (Table 5.12a). Swede appressoria were smaller on middle leaves than on other leaves; on oilseed rape, apical leaves encouraged larger appressoria compared with mid-leaves but on Brussels sprout the largest appressoria were produced on middle leaves.

E. cruciferarum preferred periclinal wall sites for formation of appressoria with an overall ratio of 8.5: 1.4: 0.1 for periclinal, anticlinal and guard cell wall areas (Fig. 5.31). A significant main effect of brassica and a significant interaction between brassica and leaf position was seen for proportions at both periclinal and anticlinal sites. Brussels sprout gave rise to fewer periclinal appressoria than either swede or oilseed rape. This was true for all leaf positions except apical leaves, where Brussels sprout had slightly higher numbers of periclinal wall appressoria than swede. Anticlinal wall appressoria were most frequent on Brussels sprout and few in numbers on oilseed rape. On apical leaves, however, swede produced more appressoria at anticlinal positions than Brussels sprout. Very few appressoria were produced around guard cells.

The proportion of appressoria which failed to successfully penetrate and

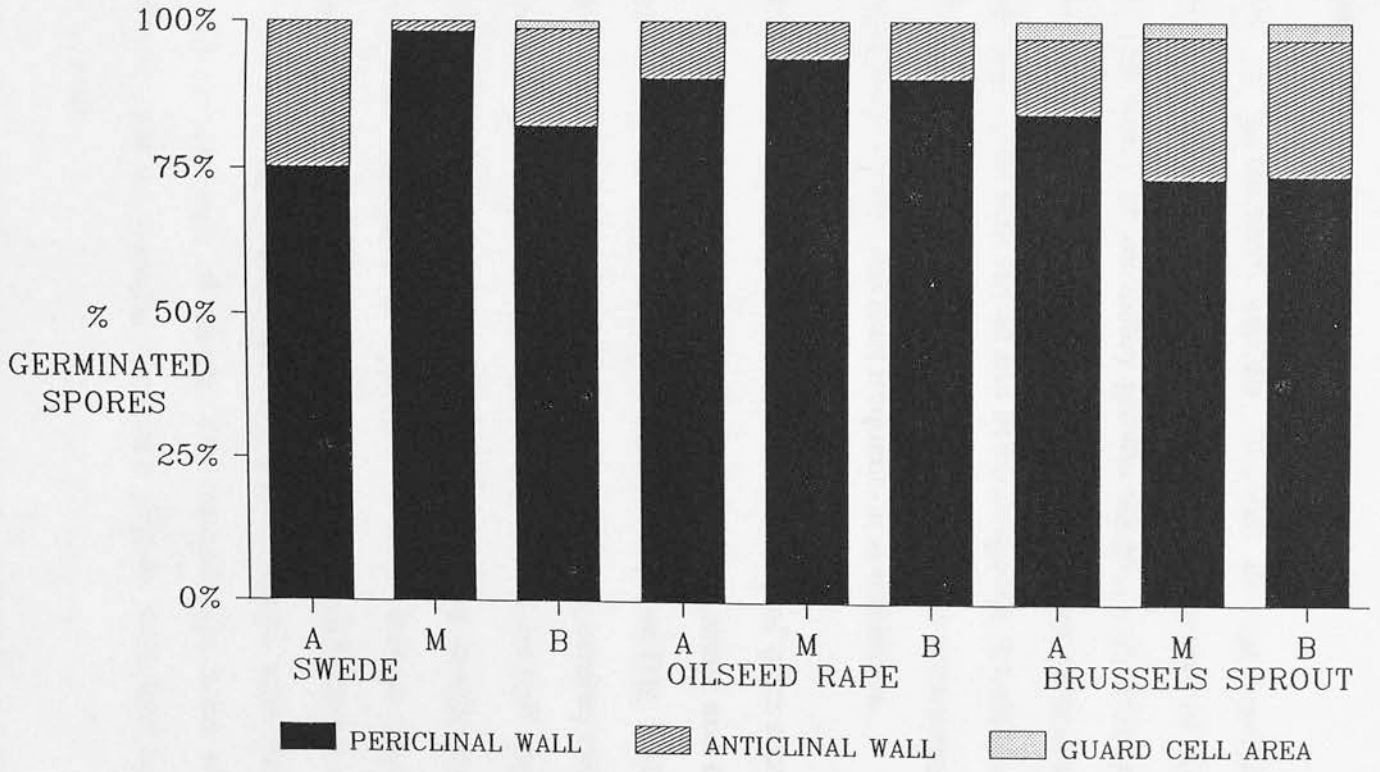
Table 5.11. Germination (%), germ-tube length and percentage germinated spores forming appressoria of Erysiphe cruciferarum in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Germination				
Swede	57.6	53.6	62.4	57.9
Oilseed Rape	54.4	52.0	58.4	54.9
Brussels sprout	54.4	59.3	55.3	56.3
Mean	55.5	55.0	58.7	56.4
(b) Appressorial germ-tube length (1 unit ≡ 45 μm)				
Swede	0.6	0.4	0.5	0.5
Oilseed Rape	0.5	0.4	0.5	0.4
Brussels sprout	0.6	0.5	0.5	0.5
Mean	0.6	0.4	0.5	0.5
(c) Numbers forming appressoria (% germinated spores)				
Swede	94.2	95.6	97.5	95.8
Oilseed Rape	98.3	98.6	95.6	97.5
Brussels sprout	96.4	98.7	97.5	97.5
Mean	96.3	97.6	96.9	96.9

SED:	(a)	(b)	(c)	d.f.
Brassica type	2.80	0.02	1.78	8
Leaf position	3.70	0.04	1.73	22
Brassica type x Leaf position	5.93	0.06	3.02	22
(at same level of brassica)	6.41	0.07	3.00	

Fig. 5.31.

Sites of hyphal termination of *E. cruciferarum* on apical (A), middle (M) and basal (B) leaves of different brassicas



SED= +/− PERICLIANL 6.44; ANTICLINAL 5.51; GUARD CELL 2.19
d.f.= 21

produce secondary structures on the various surfaces was influenced by brassica and leaf position (Table 5.12b). A significantly higher number of germinated conidia failed to establish a successful relationship with Brussels sprout compared with swede and oilseed rape. With respect to leaf position, numbers on apical and basal leaves were similar, whilst those on mid-leaves were significantly lower.

About 75% of germinated conidia formed an appressoria and successfully formed secondary structures (Table 5.12c). Analysis of variance of the results for frequency of secondary growth showed a significant main effect for brassica and leaf position. Oilseed rape was most favourable for secondary growth compared with swede and Brussels sprout, the numbers on the latter being least. Numbers of appressoria with secondary structures were more frequent on middle leaves, and least frequent on apical leaves.

Analysis of host reactions showed that in the majority of cases secondary development took place in association with a papilla, with swede and oilseed rape forming significantly more papillae than Brussels sprout (Fig. 5.32). The numbers of appressorial penetrations forming secondary structures without a papilla were generally small. Of the unsuccessful penetrations half appeared to be associated with papillae, numbers with papillae being usually most on apical and basal leaves. Numbers of appressorial sites without secondary structures or papillae were most frequent on apical leaves of Brussels sprout and on swede: on both these brassicas numbers declined with leaf age, particularly rapidly in the case of swede. On oilseed rape sites without secondary structures and no papillae increased slightly with leaf age but always tended to be low.

On its establishment with the host, *E. cruciferarum* had the potential to

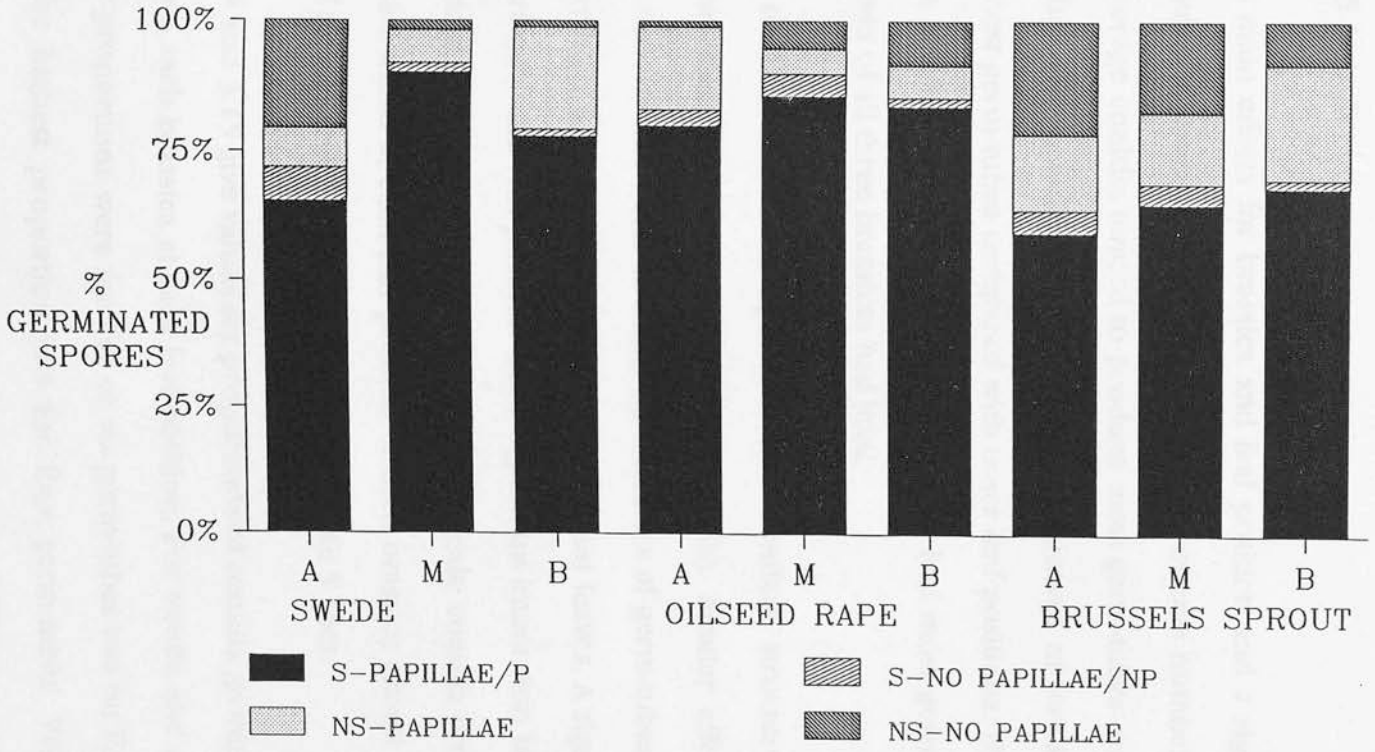
Table 5.12. Appressorial size, percentage abortive appressoria and percentage of spores forming secondary structures of Erysiphe cruciferarum in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Appressorial size (scale 1-6)				
Swede	5.3	4.7	5.3	5.1
Oilseed Rape	5.6	4.9	5.0	5.1
Brussels sprout	5.2	5.7	5.5	5.5
Mean	5.4	5.1	5.3	5.3
(b) Numbers of abortive appressoria (% germinated spores)				
Swede	28.7	7.8	20.4	19.0
Oilseed Rape	21.5	9.6	14.0	15.0
Brussels sprout	33.2	30.5	29.3	31.0
Mean	27.8	16.0	21.2	21.7
(c) Numbers forming secondary structures (% germinated spores)				
Swede	61.6	87.7	77.1	75.5
Oilseed Rape	76.8	89.0	81.6	82.5
Brussels sprout	67.6	71.5	67.2	68.8
Mean	68.7	82.7	75.3	75.6

SED:	(a)	(b)	(c)	d.f.
Brassica type	0.02	4.82	4.41	8
Leaf position	0.20	4.08	4.77	22
Brassica type x Leaf position	0.34	7.52	7.91	22
(at same level of brassica)	0.34	7.06	8.16	

Fig. 5.32.

Host papillae with (S) or without (NS) secondary structures of *E.cruciferarum* on apical (A), middle (M) and basal (B) leaves of different brassicas



SED= +/- S/P 9.54; S/NP 3.46; NS/P 6.94; NS/NP 6.33
d.f.= 21

form a germ-tube at each point of the conidium plus a germ-tube from beneath or extending from the appressorium i.e. from one to six germ-tubes (Fig. 5.1). Each of these germ-tubes could form a branch and a conidial initial could form on either germ-tube or branch. Average numbers of germ-tubes, branches and conidial initials on conidia, summarising the data overall, are given in Tables 5.13 to 5.23.

Significant main effects for brassica and leaf position and a significant interaction between brassica and leaf position for germ-tube numbers were obtained. On average conidia tended to produce most germ-tubes on swede than on the other two brassicas (Table 5.13a). Mid-leaves of swede were found to have most germ-tubes compared with other leaf positions. However for oilseed rape and Brussels sprout the basal leaves had most germ-tubes, whilst apical leaves of all three brassicas had least.

Numbers of branches from spores with secondary structures was influenced by brassica and leaf position (Table 5.13b). Similar effects for branching were seen as had been obtained for numbers of germ-tubes, more branching occurring, on average, on swede and on basal leaves. A significant main effect of brassica and leaf position and a significant interaction between the two was evident for average number of conidial initials: conidia produced about one conidial initial at each leaf position of each brassica except on the middle leaves of swede where two were produced (Table 5.13c).

Tables 5.14 and 5.15 give values for proportions of conidia giving rise to 1-6 germ-tubes on each brassica at each leaf position. For swede and oilseed rape the highest proportions were for five or six germ-tubes but on Brussels sprout leaves the highest proportion was for four germ-tubes. Very few conidia had only one or two germ-tubes on all brassicas (Table 5.14). With

Table 5.13. Average numbers of primary germ tubes, branches on germ-tubes and conidial initials on conidia of Erysiphe cruciferarum in relation to brassica type and leaf position.

BRASSICA TYPE	LEAF POSITION Apical	Middle	Basal	Mean
(a) Average number of germ-tubes*				
Swede	4.0	5.7	5.1	4.9
Oilseed Rape	4.3	4.6	5.1	4.6
Brussels sprout	3.8	3.8	4.7	4.1
Mean	4.0	4.7	5.0	4.6
(b) Average number of branches*				
Swede	2.3	3.7	3.2	3.1
Oilseed Rape	2.1	2.9	3.3	2.7
Brussels sprout	1.6	2.1	2.6	2.1
Mean	2.0	2.9	3.0	2.6
(b) Average number of conidial initials*				
Swede	1.1	2.1	1.2	1.4
Oilseed Rape	1.2	1.2	1.0	1.1
Brussels sprout	1.0	1.0	1.0	1.0
Mean	1.1	1.4	1.1	1.2

SED:	(a)	(b)	(c)	d.f.
Brassica type	0.27	0.20	0.08	8
Leaf position	0.20	0.24	0.11	2
Brassica type x Leaf position	0.38	0.39	0.18	22
(at same level (of brassica)	0.39	0.41	0.20	

* - on germinated spores forming secondary structures

Table 5.14. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on number of germ-tubes produced in relation to brassica type (averaged for leaf position).

BRASSICA TYPE	NUMBER OF GERM-TUBES PRODUCED (%)					
	1	2	3	4	5	6
Swede	2.6	4.2	5.0	15.9	27.8	44.0
Oilseed Rape	3.1	7.3	4.9	25.3	29.6	30.6
Brussels Sprout	6.7	9.0	10.8	30.3	26.7	16.5
SED= +/- (d.f.=22)	3.08	4.02	4.75	3.30	5.38	7.56

Table 5.15. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on number of germ-tubes produced in relation to leaf position (averaged for brassica type).

LEAF POSITION	NUMBER OF GERM-TUBES PRODUCED (%)					
	1	2	3	4	5	6
Apical	4.2	12.7	15.3	27.4	25.2	15.2
Middle	5.8	6.4	3.3	21.2	25.7	38.3
Basal	2.3	1.5	2.1	22.8	33.3	37.6
SED= +/- (d.f.=22)	2.50	3.21	3.59	5.58	4.38	5.31

respect to leaf position, on mid- and basal leaves the highest proportions gave six germ-tubes whilst on apical leaves most conidia had four or five germ-tubes (Table 5.15). Once again very few conidia were seen to have only one or two germ-tubes on all leaves, but particularly basal leaves. Generally relative germ-tube numbers were not influenced by brassica and only in some cases by leaf position but numbers of spores with six germ-tubes showed a significant main effect for brassica, leaf position and the interaction between the two factors (Fig. 5.33). Mid-leaves of swede were seen to have largest proportions of spores with six germ-tubes. With oilseed rape and Brussels sprout numbers of conidia with six germ-tubes increased with leaf age but the numbers on Brussels sprout always tended to be comparatively low. Apical leaves of all three brassicas had the smallest numbers of conidia with six germ-tubes.

The proportion of germ-tubes originating at each conidial position (Fig. 5.1) is shown in Tables 5.16 and 5.17. Almost all conidia produced a germ-tube at the C and A positions for all three brassicas at all leaf positions. D and B positions also produced germ-tubes frequently but showed variation with regard to brassica and leaf position: as a rule those leaves which least supported secondary development, *i.e.* Brussels sprouts and apical leaves, had fewest germ-tubes originating at the D and B positions. Positions X and Y overall had the lowest frequencies of germ-tubes. Swede showed highest numbers at X and Y and Brussels sprout the least. Apical leaves tended to have comparatively low values for both X and Y positions. Germ-tubes at the X position were mostly prevalent on mid-leaves but Y germ-tubes were most abundant on basal leaves.

The interaction between brassica and leaf position in relation to

Table 5.16. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on position of germ-tubes in relation to brassica type (averaged for leaf position).

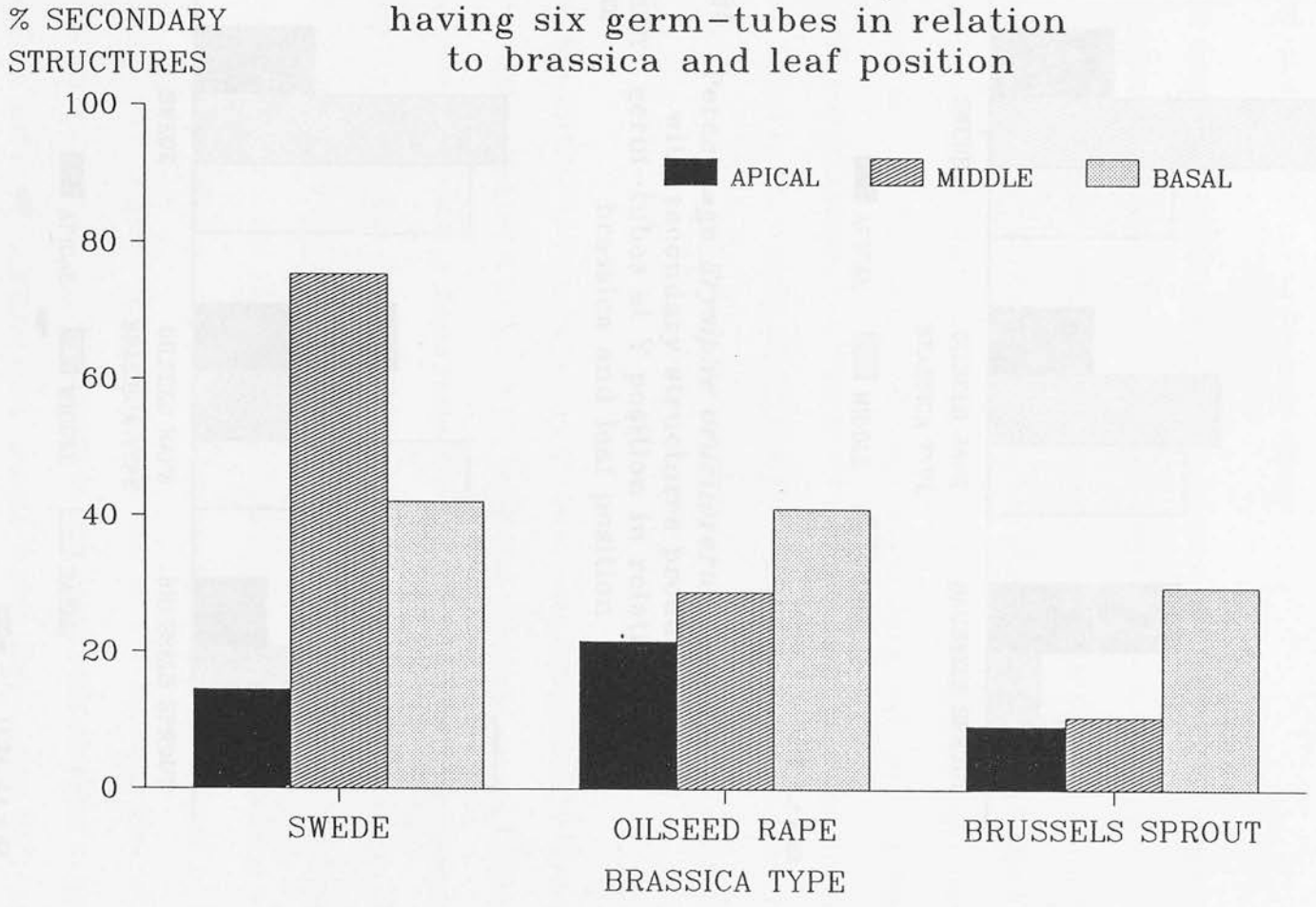
BRASSICA TYPE	POSITION OF GERM-TUBES (%)					
	C	A	D	B	X	Y
Swede	94.2	99.3	85.7	92.6	56.3	61.2
Oilseed Rape	94.7	92.7	87.6	84.5	46.4	57.0
Brussels Sprout	89.8	96.0	78.9	73.4	33.9	48.7
SED= +/- (d.f.=22)	4.19	2.74	6.02	8.61	7.48	7.92

Table 5.17. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on position of germ-tubes in relation to leaf position (averaged for brassica type).

LEAF POSITION	POSITION OF GERM-TUBES (%)					
	C	A	D	B	X	Y
Apical	90.0	93.1	72.0	77.2	36.8	34.4
Middle	90.9	95.3	87.2	82.9	54.1	58.7
Basal	97.9	99.7	92.9	90.4	45.8	73.8
SED= +/- (d.f.=22)	2.50	2.20	5.00	5.10	8.02	5.64

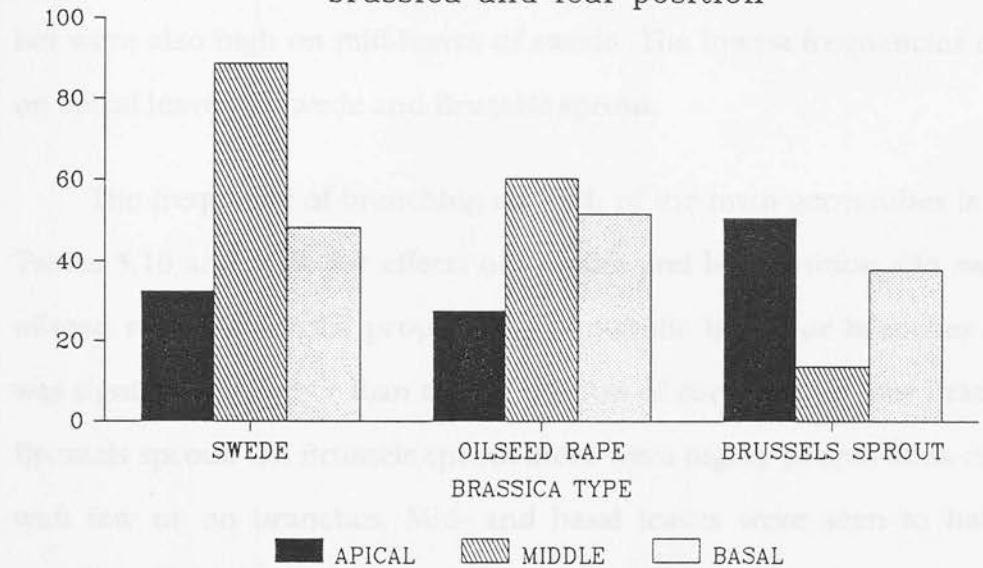
Fig. 5.33.

Percentage of conidia of *Erysiphe cruciferarum* with secondary structures having six germ-tubes in relation to brassica and leaf position



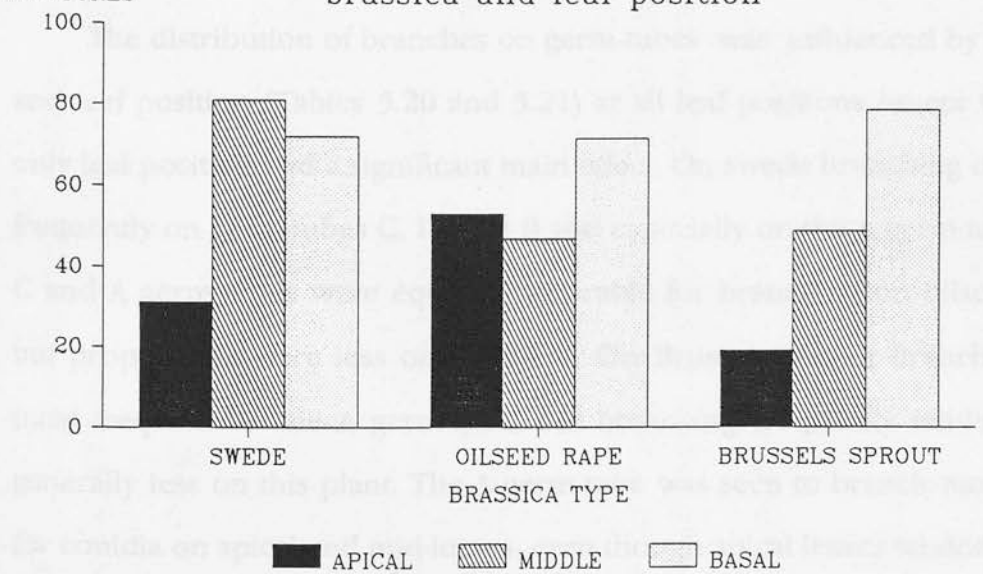
SED= +/- 10.65; d.f.= 22

Fig. 5.34. Percentage *Erysiphe cruciferarum* conidia with secondary structures producing germ-tubes at X position in relation to brassica and leaf position



SED= +/- 13.58; d.f.= 22

Fig. 5.35. Percentage *Erysiphe cruciferarum* conidia with secondary structures producing germ-tubes at Y position in relation to brassica and leaf position



SED= +/- 11.24; d.f.= 22

production of germ-tubes at X and Y positions is shown in Figs 5.34 and 5.35. In the case of X, proportions were highest on mid-leaves and lowest on apical leaves for swede and oilseed rape, but the reverse applied for Brussels sprout. For Y germ-tubes, numbers were high on basal leaves of all brassicas but were also high on mid-leaves of swede. The lowest frequencies occurred on apical leaves of swede and Brussels sprout.

The frequency of branching on each of the main germ-tubes is given in Tables 5.18 and 5.19, for effects of brassica and leaf position. On swede and oilseed rape the largest proportions of conidia had four branches and this was significantly higher than the proportion of conidia with four branches on Brussels sprout. On Brussels sprout there were higher proportions of conidia with few or no branches. Mid- and basal leaves were seen to have more conidia with higher numbers of branches, highest proportions being for four branches. Apical leaves had significantly fewer conidia with four branches but showed significantly higher proportions of conidia with no branches compared with other leaves.

The distribution of branches on germ-tubes was influenced by brassica and leaf position (Tables 5.20 and 5.21) at all leaf positions except C where only leaf position had a significant main effect. On swede branching occurred frequently on germ-tubes C, D, and B and especially on the A germ-tube. The C and A germ-tubes were equally favourable for branching on oilseed rape but proportions were less on D and B. On Brussels sprout branching was most frequent on the A germ-tube, but branching frequently tended to be generally less on this plant. The A germ-tube was seen to branch more often for conidia on apical and mid-leaves, even though apical leaves tended to have fewer conidia with branches than mid- or basal leaves. Basal leaves had most

Table 5.18. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on the number of branches produced in relation to brassica type (averaged for leaf position).

BRASSICA TYPE	NUMBERS OF BRANCHES PRODUCED				
	0	1	2	3	4
Swede	9.8	12.6	7.6	9.9	59.5
Oilseed rape	18.4	8.8	16.0	18.0	40.1
Brussels sprout	31.2	17.7	15.1	15.3	20.7
SED= +/- (d.f.=22)	7.68	3.96	2.83	4.70	6.20

Table 5.19. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on the number of branches produced in relation to leaf position (averaged for brassica type).

LEAF POSITION	NUMBER OF BRANCHES PRODUCED				
	0	1	2	3	4
Apical	33.0	19.6	15.3	11.1	20.4
Middle	16.3	5.4	14.1	18.4	45.7
Basal	10.1	14.0	9.3	13.7	54.3
SED= +/- (d.f.=22)	5.93	4.65	4.13	4.05	8.21

Table 5.20. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on the position of branches in relation to brassica type (averaged for leaf position).

BRASSICA TYPE	POSITIONS OF BRANCHES (%)				
	0*	C	A	D	B
Swede	9.8	74.3	81.3	73.4	68.1
Oilseed rape	18.4	72.1	71.0	54.9	46.3
Brussels sprout	31.2	48.5	57.2	37.8	32.0
SED = +/- (d.f. = 22)	7.68	10.05	7.05	5.73	9.92

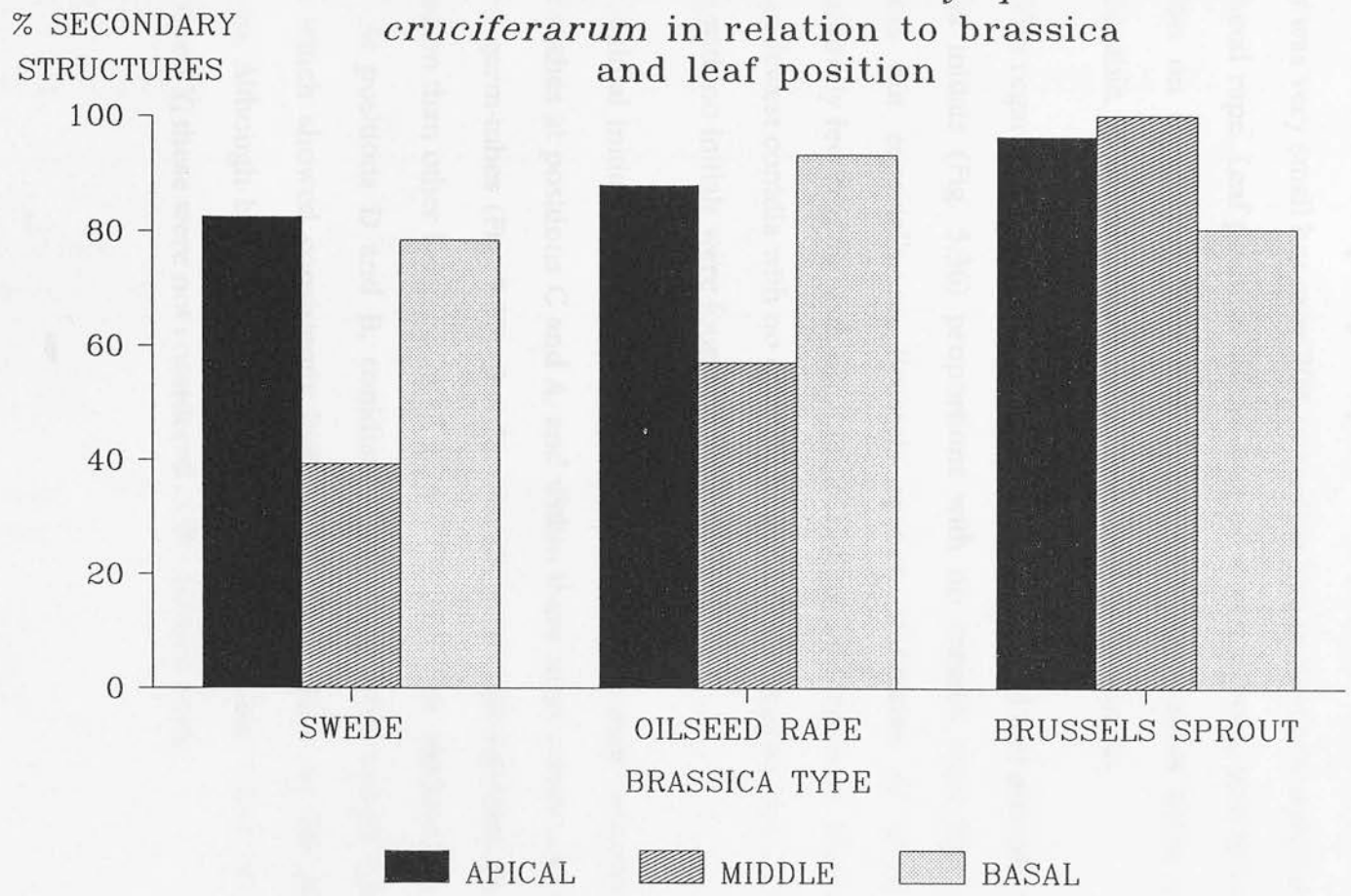
Table 5.21. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on the position of branches in relation to leaf position (averaged for brassica type).

LEAF POSITION	POSITION OF BRANCHES (%)				
	0*	C	A	D	B
Apical	33.0	46.9	58.0	31.7	31.1
Middle	16.3	66.0	75.3	68.7	58.2
Basal	10.1	81.9	76.3	65.7	57.2
SED = +/- (d.f. = 22)	5.93	9.95	6.56	7.75	7.80

* - no branching

Fig. 5.36.

Percentage of secondary structures with no conidial initials for *Erysiphe cruciferarum* in relation to brassica and leaf position



SED= +/- 9.36; d.f.= 22

conidia with branches on the C germ-tube.

Conidiation occurred infrequently on all brassicas except swede (Tables 5.22 and 5.23). Thus oilseed rape and particularly Brussels sprout had a significantly higher proportion of conidia with no conidial initials compared with this brassica. Frequency of spores with conidial initials on Brussels sprout was very small but over 20% of conidia had at least one conidial initial on oilseed rape. Leaf position effects showed conidia production to be most common on mid-leaves, with both apical and basal leaves being equally unfavourable. Most spores gave rise to only one conidial initial.

With regard to the interaction between brassica and leaf position for no conidial initials (Fig. 5.36) proportions with no conidia were high on all brassicas but especially on Brussels sprout. Mid-leaves of swede had comparatively few conidia with no conidial initials. Mid-leaves of oilseed rape also had fewest conidia with no conidial initials but on Brussels sprout fewest spores with no initials were found on basal leaves.

Conidial initials were found to occur most commonly on germ-tubes and branches at positions C and A, and within these most commonly on the primary germ-tubes (Fig. 5.37). Swede, overall, promoted significantly more conidiation than other brassicas at all germ-tube positions, especially on mid-leaves. At positions D and B, conidiation was infrequent except again on swede which showed consistently high conidiation values on the mid-leaf positions. Although branching and conidial initiation could occur on germ-tubes X and Y, these were not considered in the present study.

Table 5.22. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on the number of conidial initials produced in relation to brassica type (averaged for leaf position).

BRASSICA TYPE	NUMBER OF CONIDIAL INTITIALS (%)				
	0	1	2	3	4
Swede	66.7	9.8	9.5	3.8	9.5
Oilseed rape	79.4	19.9	3.8	1.2	0.0
Brussels sprout	92.4	5.0	1.7	0.0	0.0
SED= +/- (d.f.= 22)	4.73	4.86	2.11	1.89	2.31

Table 5.23. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures in different categories based on the number of conidial initials produced in relation to leaf position (averaged for brassica type).

LEAF POSITION	NUMBER OF CONIDIAL INITIALS (%)				
	0	1	2	3	4
Apical	88.9	12.8	3.4	0.6	0.0
Middle	65.2	14.3	5.8	4.5	8.9
Basal	84.3	7.6	5.8	0.0	0.5
SED= +/- (d.f.= 22)	5.38	5.43	2.78	2.10	2.88

Fig. 5.37. Percentage of conidial initials on germ-tubes and branches of *Erysiphe cruciferarum* in relation to brassica and leaf position (% secondary structures)

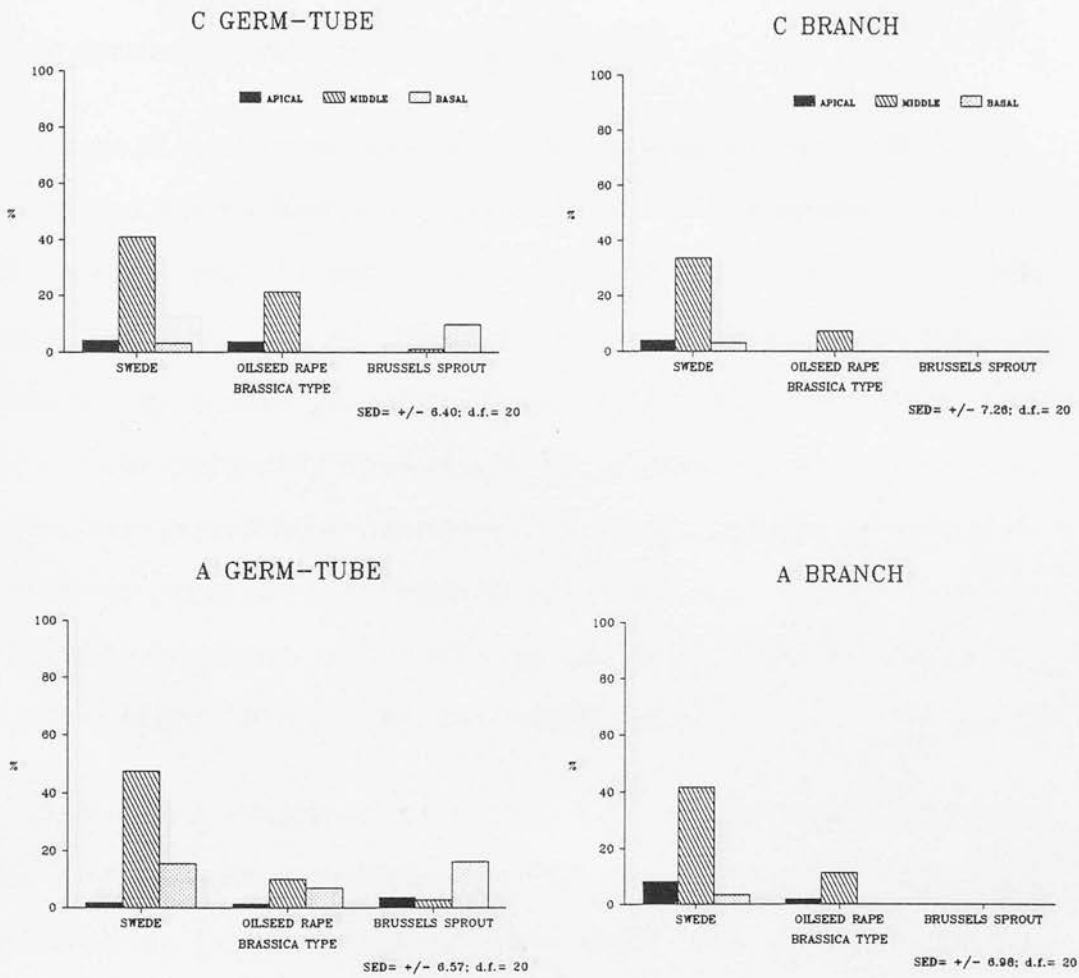
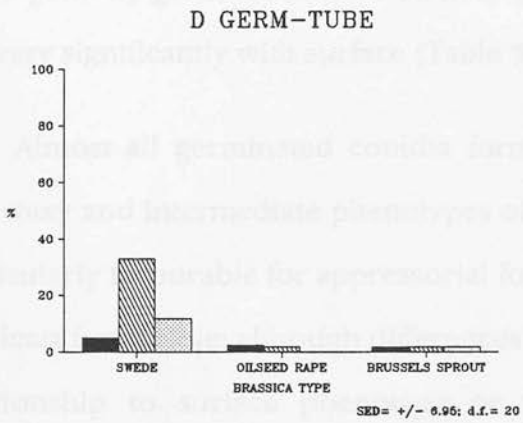
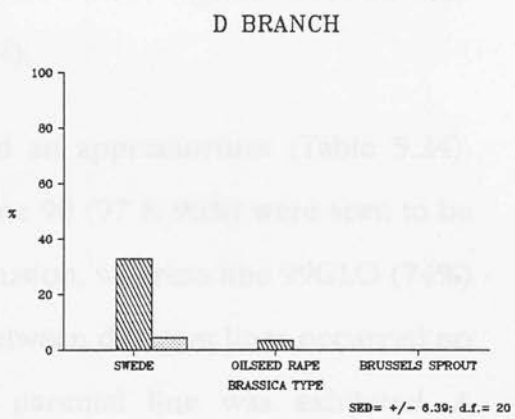
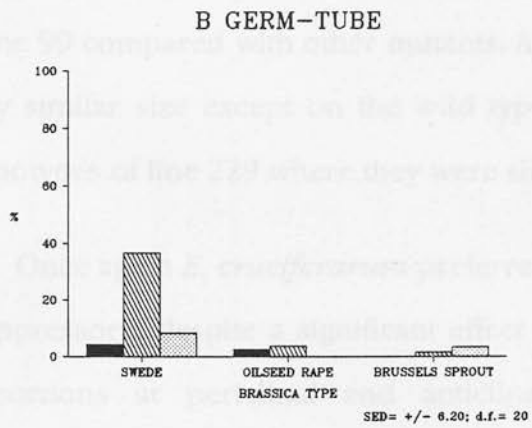
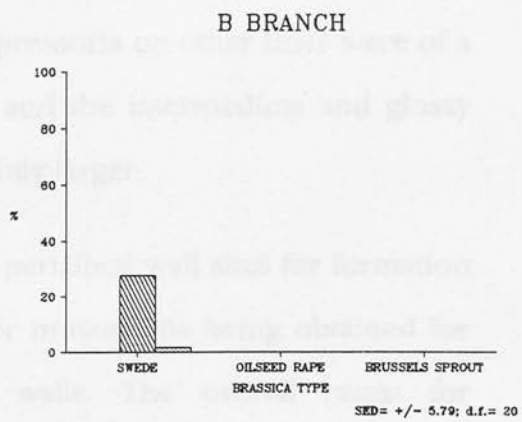


Fig 5.37. continued.









5.3.4. Leaf surface behaviour and early penetration events of **Erysiphe cruciferarum** *in relation to* **gemmifera** *mutant line*

The analyses of variance of the data for this experiment are summarised in Appendix 5.7. A significant effect of mutant line was seen for germination which ranged from 58-83% (Table 5.24). Parental line 90 generally allowed a higher rate of germination than line 229, 99GLO or the wild type. The lengths of the primary germ-tubes were on average one unit or slightly above and did not vary significantly with surface (Table 5.24).

Almost all germinated conidia formed an appressorium (Table 5.24). The waxy and intermediate phenotypes of line 90 (97 & 96%) were seen to be particularly favourable for appressorial formation, whereas line 99GLO (74%) was least favourable: although differences between different lines occurred no relationship to surface phenotype or to parental line was exhibited. A significant effect of mutant line was seen for appressorial size (Table 5.25), this being mainly associated with unusually small appressoria found on leaves of line 99 compared with other mutants. Appressoria on other lines were of a fairly similar size except on the wild type and the intermediate and glossy phenotypes of line 229 where they were slightly larger.

Once again *E. cruciferarum* preferred periclinal wall sites for formation of appressoria despite a significant effect for mutant line being obtained for proportions at periclinal and anticlinal walls. The overall ratios for appressorial sites were 9.0: 0.9: 0.1 for periclinal, anticlinal and guard cell areas (Fig. 5.38). The waxy and glossy phenotypes of line 229 plus line 99GLO had relatively low incidences of periclinal appressoria (84, 76 and 74%) corresponding to their relatively high incidences of anticlinal wall appressoria. In contrast the intermediate phenotype of line 229 and the waxy

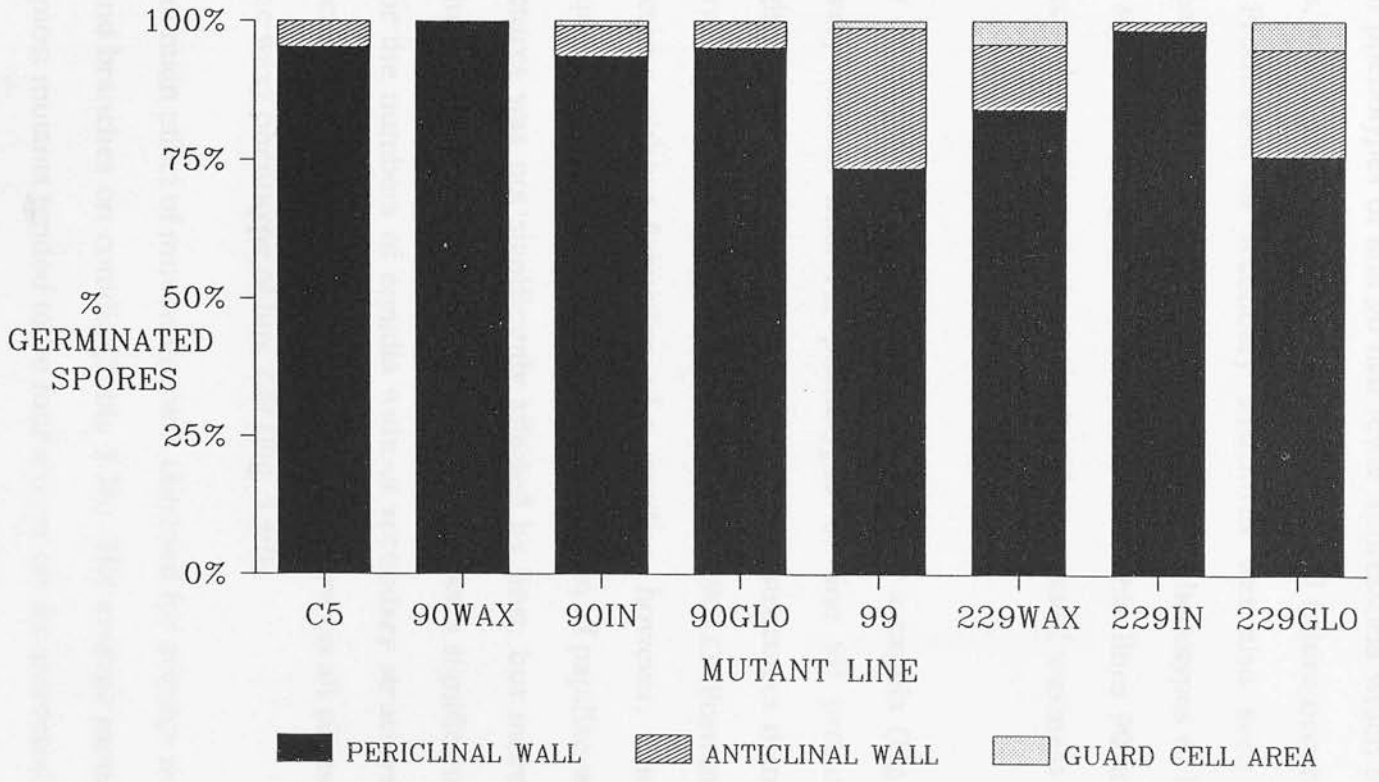
Table 5.24. Germination rate, appressorial germ-tube length and percentage geminated spores forming appressoria of Erysiphe cruciferarum on leaves of gemmifera mutants.

MUTANT LINE	Germination (%)	Appressorial germ-tube length *	Numbers of appressoria (% gs)
C5	64.0	1.2	80.1
90WAX	83.2	1.0	97.2
90INT	82.4	1.1	96.2
90GLO	71.2	1.1	86.9
99GLO	58.4	1.2	74.3
229WAX	68.8	1.1	88.4
229INT	56.8	1.1	86.1
229GLO	72.8	1.4	91.8
SED = +/- (d.f. = 28)	6.71	0.11	5.05

gs- germinated spores *- 1 unit ≡ 45 μm

Fig. 5.38.

Sites of hyphal termination of *Erysiphe cruciferarum* in relation to *gemmifera* mutant line



SED= +/− PERICLINAL 6.86; ANTICLINAL 7.75; GUARD CELL 4.10
d.f.= 26

phenotype of line 90 had periclinal appressoria, in more or less all cases, but no significant relationships with either surface type or parental line were evident. Appressoria formed around guard cells occurred only very rarely.

Abortive appressorial frequency seemed to be affected by parental line (Table 5.25). All phenotypes of line 90 had fewer appressoria which aborted than other lines, whilst particularly frequent appressorial failure occurred on line 229GLO. Production of secondary structures was also seen to be influenced by parental line (Table 5.25). Numbers on phenotypes of line 90 were generally significantly higher than other lines. Within lines 90 and 229 frequency of secondary structures increased with increased waxiness of the surface.

Secondary development was often associated with a papilla (Fig 5.39), but not for every mutant line. All phenotypes of line 90 produced a significantly higher number of papillae with secondary structures than other mutants while relatively few were produced on line 229GLO. Formation of secondary structures without formation of a papillae, however, tended to occur more frequently on 229 lines (Fig. 5.39). Formation of papillae without secondary structures was not significantly affected by host, but more were seen to be produced on phenotypes of line 229. In contrast a significant effect was obtained for the numbers of conidia without secondary structures and without an associated papilla. Numbers were relatively low on all phenotypes of line 90 and the waxy phenotype of line 229 (Fig. 5.39).

A significant main effect of mutant line was obtained for average number of germ-tubes and branches on conidia (Table 5.26). The average number of germ-tubes for most mutants tended to be four except on the intermediate of line 229 where three were produced and the glossy of line 229 where the

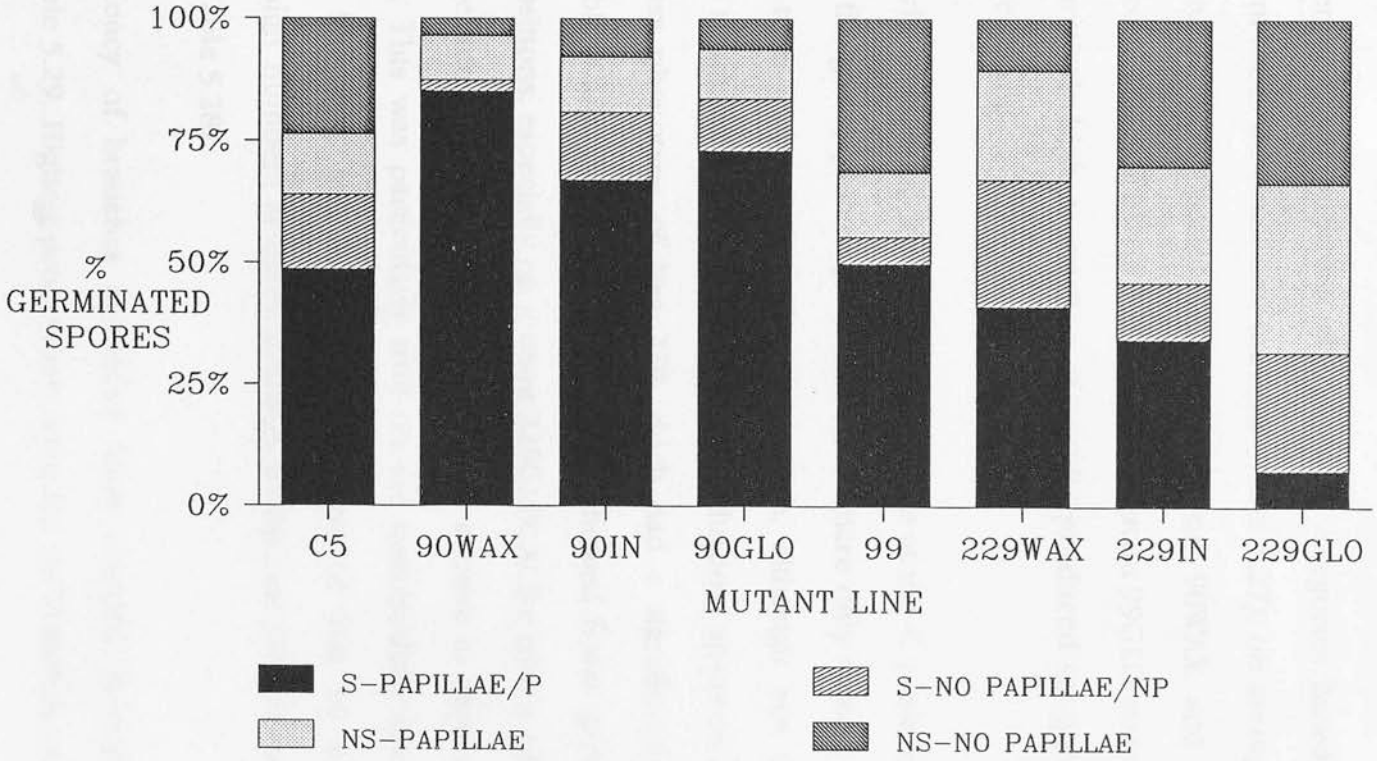
Table 5.25. Appressorial size, percentage abortive appressoria of geminated spores and percentage germinated spores forming secondary structures of Erysiphe cruciferarum on leaves of gemmifera mutants.

MUTANT LINE	Appressorial size (scale 1-6)	Abortive appressoria (% gs)	Secondary structures (% gs)
C5	5.7	24.7	48.2
90WAX	4.5	13.3	69.1
90INT	4.6	18.5	60.6
90GLO	5.0	14.5	58.5
99GLO	3.4	29.3	40.6
229WAX	4.8	30.4	50.1
229INT	5.7	45.0	38.0
229GLO	5.7	61.0	30.5
SED= +/- (d.f.= 28)	0.29	7.11	5.49

gs- geminated spores

Fig. 5.39.

Host papillae with (S) or without (NS) secondary structures of *E. cruciferarum* in relation to *gemmifera* mutants



SED= +/- S/P 10.06; S/NP 7.40; NS/P 8.29; NS/NP 7.52
d.f.= 26

average number was two. On average branching occurred once on each conidium with secondary structures except on the waxy phenotype of line 90 and line 99GLO where slightly higher frequencies were seen. The glossy phenotype of line 229 tended to have conidia with slightly fewer branches compared with other mutants.

In considering the proportions of conidia in categories based on the numbers of germ-tubes on conidia of mutants (Table 5.27), on average three or four germ-tubes were produced. However, on 90WAX and 99GLO numbers reached five in more than 30% of cases. Mutant 99GLO was unusual in that a comparatively high proportion of conidia produced six germ-tubes and few produced three.

All or nearly all conidia produced a germ-tube at the C position on all mutants except the glossy phenotype of line 229, where only 80% of conidia had a C germ-tube (Table 5.28). The A position, although not quite as favourable, had a high frequency of germ-tube production, again on all lines except the glossy phenotype of line 229 which had a significantly lower proportion (Table 5.28). The D and B positions showed fewer germ-tubes than C and A positions, especially on mutant 229GLO. At the minor positions, i.e. X and Y, the numbers of germ-tubes were low relative to those seen at other positions. This was particularly true on the intermediate and glossy phenotypes of line 229, but the waxy phenotype of line 90 still had comparatively high numbers at the X position, whilst line 99GLO had high numbers at Y (Table 5.28).

The frequency of branches produced from conidial germ-tubes is indicated in Table 5.29. Highest proportions were for no branches except on

Table 5.26. Average number of germ-tubes and branches on germ-tubes of Erysiphe cruciferarum on conidia on leaves of different gemmifera mutants.

MUTANT LINE	Average number of germ-tubes*	Average number of branches*
C5	3.5	1.1
90WAX	4.3	1.3
90IN	3.8	1.2
90GLO	3.6	1.1
99GLO	4.4	1.8
229WAX	3.5	1.1
229IN	3.2	1.1
229GLO	2.4	0.7
SED = +/- (d.f. = 28)	0.39	0.15

* - on germinated spores forming secondary structures

Table 5.27. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures from different categories based on number of in relation to gemmifera mutants.

MUTANT LINE	NUMBERS OF GERM-TUBES (%)					
	1	2	3	4	5	6
C5	0.0	15.3	29.1	29.7	19.5	2.9
90WAX	0.0	0.0	13.3	45.4	31.4	8.8
90IN	0.0	3.7	30.4	38.9	13.2	10.1
90GLO	5.0	13.1	21.6	40.2	20.1	0.0
99GLO	2.9	6.7	4.9	34.4	32.2	19.0
229WAX	1.8	10.1	37.2	41.5	9.3	0.0
229IN	0.0	22.0	38.7	35.3	4.0	0.0
229GLO*	6.7	14.2	32.4	26.7	0.0	0.0
SED = +/- (d.f. = 28)	3.08	8.44	9.25	11.34	8.53	6.37

* note: % of conidia not producing secondary structures not included in table.

Table 5.28. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures from different categories based on position in relation to gemmifera mutants.

MUTANT LINE	POSITIONS OF GERM-TUBES (%)					
	C	A	D	B	X	Y
C5	100.0	92.7	59.0	73.0	17.1	28.6
90WAX	100.0	100.0	86.6	83.3	40.6	27.1
90IN	96.8	95.2	66.1	84.7	27.3	28.0
90GLO	100.0	83.6	77.8	69.6	15.7	12.2
99GLO	100.0	94.3	85.6	85.6	21.0	43.0
229WAX	100.0	94.5	58.9	70.1	22.7	5.6
229IN	100.0	100.0	63.3	56.7	4.0	4.0
229GLO	80.0	58.4	53.3	37.1	4.2	6.7
SED= +/- (d.f.=28)	10.18	10.32	11.78	9.58	10.24	10.61

mutant 99GLO where a relatively high frequency of branching was seen. On those conidia with germ-tubes which did produce branches the number tended to be one or two. Exceptionally three or four branches were produced, but this related mainly to lines 99GLO and 229WAX. Positions of branches were variable, but it seemed the A position was especially favourable on all mutants, whilst frequency of branching at the C, D and B positions depended on each individual mutant with no clear patterns evident (Table 5.30). Once again branching and conidial initiation could occur on germ-tubes X and Y, but the numbers are not included in the table.

Table 5.29. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures from different categories based on number of branches in relation to gemmifera mutants.

MUTANT LINE	NUMBERS OF BRANCHES (%)				
	0	1	2	3	4
C5	55.4	29.8	12.0	0.0	0.0
90WAX	40.7	34.8	20.0	3.2	1.2
90IN	42.4	40.5	16.8	0.0	0.0
90GLO	56.8	30.6	11.2	1.4	0.0
99GLO	19.9	30.4	28.3	9.0	12.4
229WAX	58.5	35.8	0.0	5.6	0.0
229IN	71.3	22.7	6.0	0.0	0.0
229GLO*	55.3	24.7	0.0	0.0	0.0
SED = +/- (d.f. = 28)	13.44	10.05	7.43	2.83	3.17

* note: % of conidia not producing secondary structures not included in table.

Table 5.30. Development of Erysiphe cruciferarum: percentage of conidia with secondary structures from different categories based on position of branches in relation to gemmifera mutants.

MUTANT LINE	POSITIONS OF BRANCHES				
	0*	C	A	D	B
C5	55.4	12.0	35.1	1.5	1.5
90WAX	40.7	11.4	52.6	12.7	16.3
90IN	42.4	3.6	31.7	27.5	6.0
90GLO	56.8	12.4	35.9	10.8	5.8
99GLO	19.9	38.3	59.1	23.9	36.2
229WAX	58.5	13.1	28.5	1.8	5.6
229IN	71.3	0.0	22.0	6.0	6.7
229GLO	45.3	10.7	12.0	0.0	2.0
SED= +/- (d.f.=28)	13.44	7.03	12.28	7.56	6.27

* - no branching

5.3.5. Assessment of **Erysiphe cruciferarum** *and* **Erysiphe graminis** *development on host and non-host leaves.*

The analyses of variance of the data for this experiment is presented in Appendix 5.8. No significant differences in germination of *Erysiphe cruciferarum* on swede and barley were obtained. Germination was slightly above 70% on both surfaces (Table 5.31). The length of the appressorial germ tube was similar on both hosts (Table 5.31; Figs 5.40, 5.41, 5.42 and 5.43), with very few germ-tubes aborting before appressorial formation on both surfaces (Table 5.31). On swede 86% of appressoria developed normally but only 10% on barley leaves (Table 5.31). As found in the previous experiment *E. cruciferarum* preferred the periclinal wall locations for appressoria with 76% of total structures being formed here (Fig. 5.44). The rest were located mainly at anticlinal walls. This compared with 50% at barley leaf periclinal walls, 43% at anticlinal walls and 5% at other sites (Fig. 5.44).

If penetration of the surface by these appressoria was successful then a haustorial initials were produced. These either developed into a "normal" haustoria mainly associated with papillae (Figs 5.45, 5.46 and 5.47), or the papillae extended and the invasive structure became "encapsulated" in host wall material (Figs 5.45 and 5.48).

About 70% of attempted penetrations resulted in normal haustoria with an associated papillae in swede leaves. Thereafter, these formed secondary structures (Table 5.31) as did those few normal haustoria formed without an associated papilla (4%) (Table 5.31) A small percentage of haustoria in swede were encapsulated and some sites with

Table 5.31. Development of Erysiphe cruciferarum and Erysiphe graminis on a host and non-host plant.

PATHOGEN	HOST PLANT		SED
	Swede	Barley	(d.f. = 6)
Erysiphe cruciferarum			
(a) Germination*	74.7	71.0	5.60
(b) Germ-tube length#	0.5	0.7	0.08
(c) Abortive germ-tubes**	0.7	1.7	0.89
(d) Normal appressoria**	86.2	9.6	5.97
(e) Secondary structures**	74.0	0.0	3.19
Erysiphe graminis			
(a) Germination*	80.7	87.4	2.17
(b) 'Primary' germ-tube only	1.2	0.9	0.18
(c) Germ-tube length#	0.4	0.3	0.02
(d) Abortive germ-tubes**	17.1	1.0	2.67
(e) Normal appressoria**	73.4	95.2	5.22
(f) Secondary structures**	0.0	20.1	4.31

\# - 1 unit } 45 μm

* - % spores

** - % germinated spores

Figs 5.40 (fluorescent micrograph; x1000) - 5.43. (SEMs).

Abnormal appressorium formation of *Erysiphe cruciferarum* on barley leaves.

Fig. 5.40.

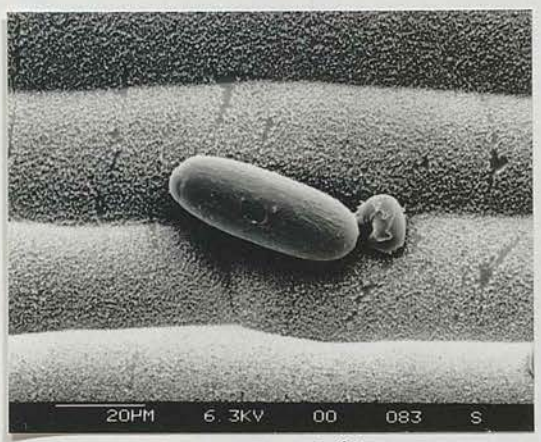


Fig. 5.41.

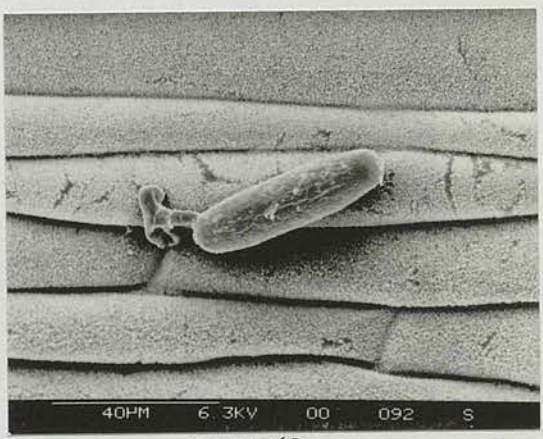


Fig 5.42.

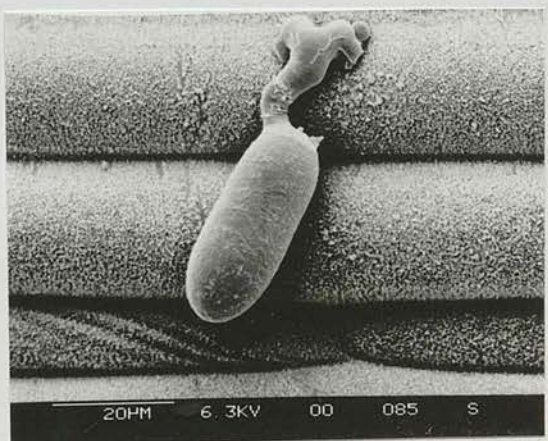


Fig. 5.43.

Figs 5.46. - 5.48. Host cell reaction of barley leaves to attempted penetrations by *Erysiphe cruciferarum.*

Fig. 5.46. Papillae and halo formation. Stained with aniline blue/trypan blue (x 400 magnification).

Fig. 5.47. Papillae formation. Stained with aniline blue (x 400 magnification).

Fig. 5.48. Encapsulation of invading structure. Stained with aniline blue (x 400 magnification).

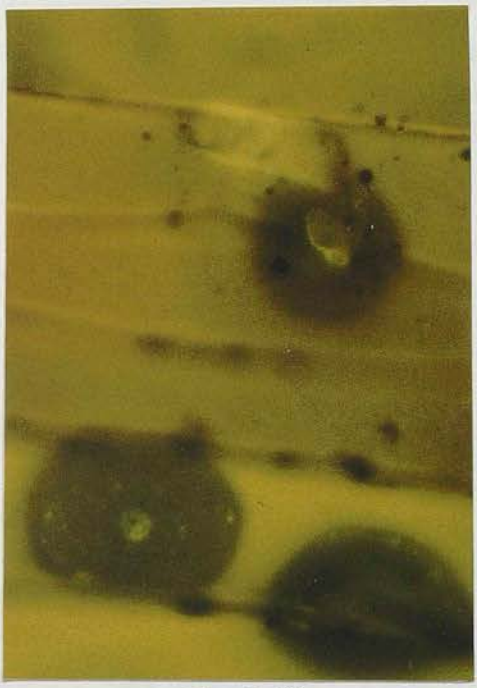
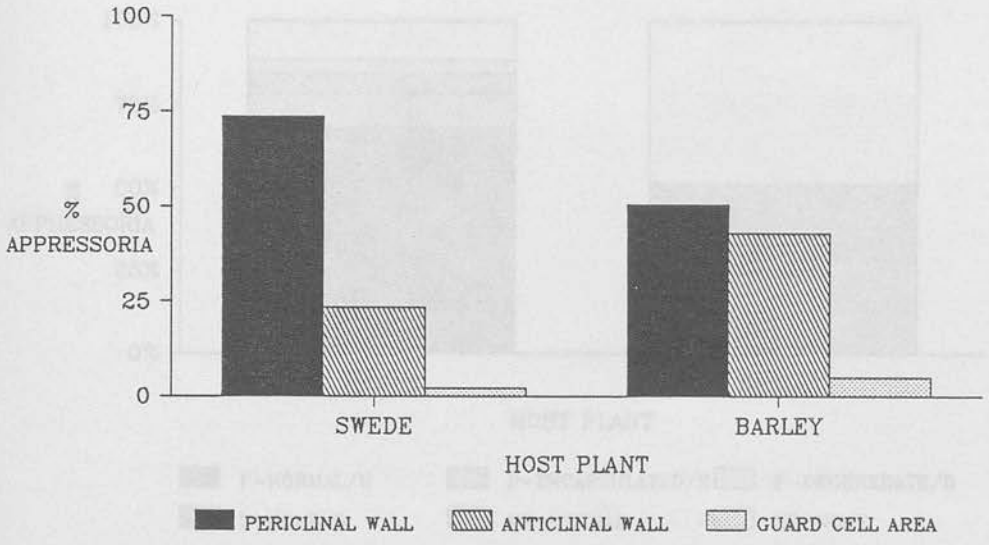


Fig. 5.46.

Fig. 5.47.

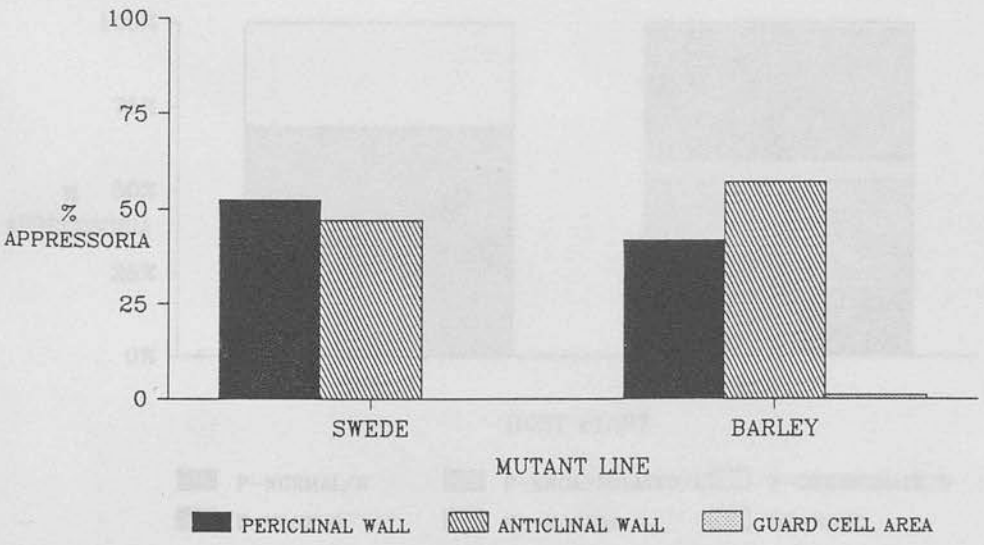
Fig. 5.48.

Fig. 5.44. Sites of hyphal termination of *Erysiphe cruciferarum* on leaves of swede and barley



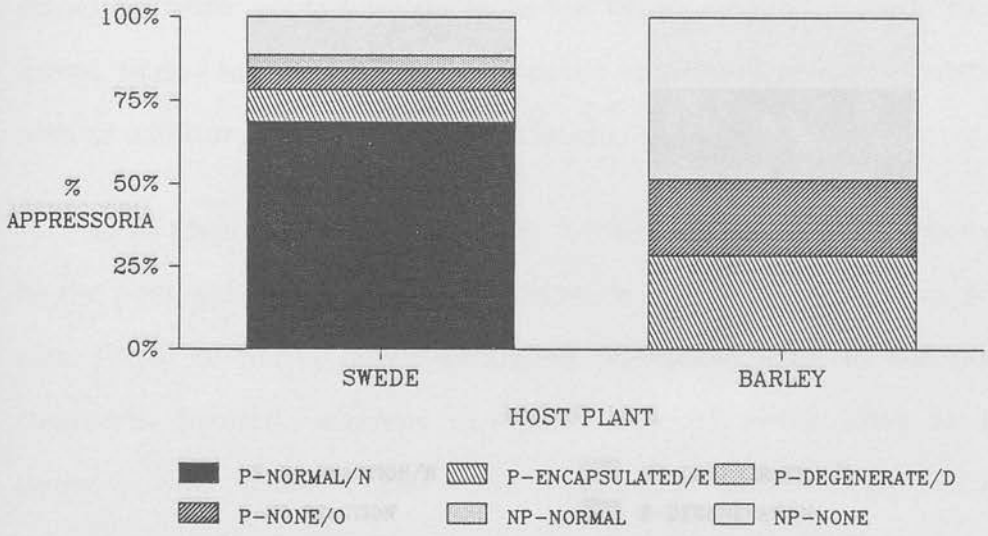
SED= +/- PERICLINAL 7.23; ANTICLINAL 9.24; GUARD CELL 2.30
d.f.= 6

Fig. 5.54. Sites of hyphal termination of *Erysiphe graminis* on leaves of swede and barley



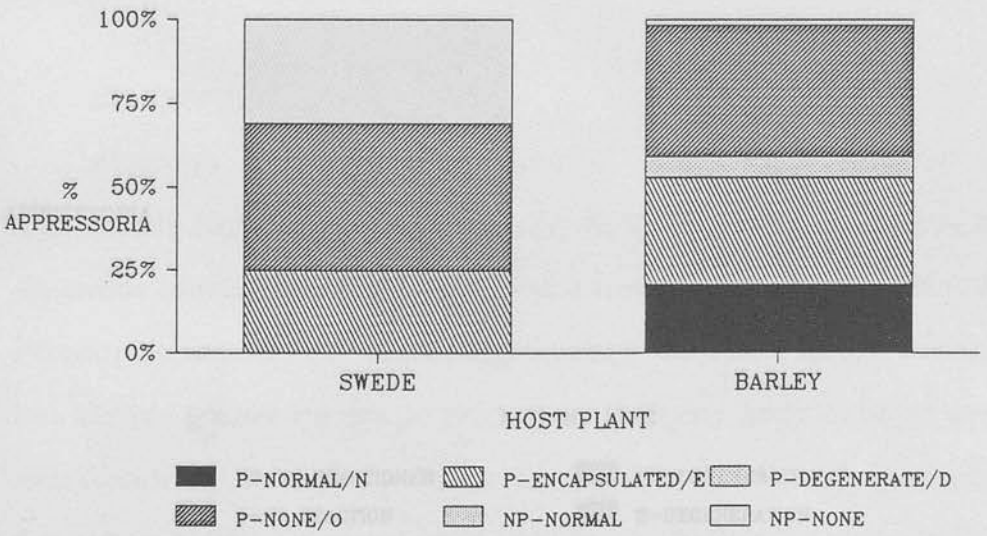
SED= +/- PERICLINAL 11.25; ANTICLINAL 11.01; GUARD CELL
d.f.= 6

Fig. 5.45. Development of haustoria in presence (P) or absence (NP) of papillae of *Erysiphe cruciferarum* on swede and barley



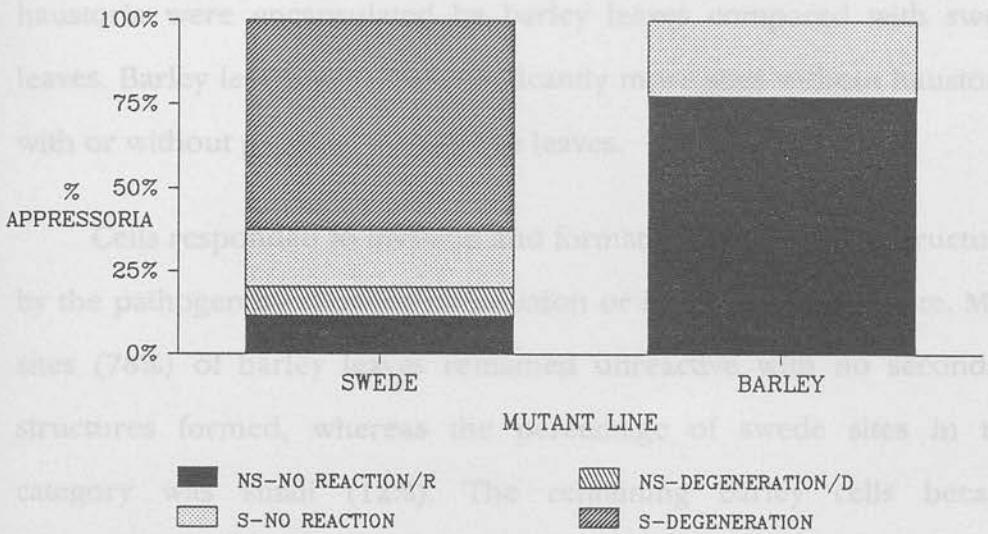
SED= +/- P/N 7.29; P/E 6.65; P/D 0.00; P/O; 7.16
NP/N 1.85; NP/O 4.80
d.f.= 6

Fig. 5.58. Development of haustoria in presence (P) or absence (NP) of papillae of *Erysiphe graminis* on swede and barley



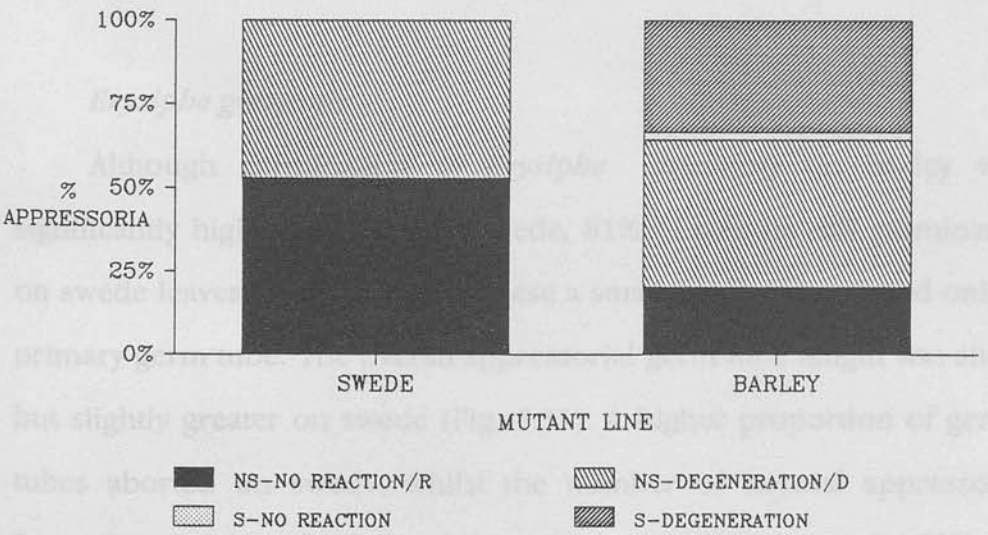
SED= +/- P/N 3.22; P/E 7.21; P/D 3.47; P/O; 10.79
NP/N 0.00; NP/O 4.22
d.f.= 6

Fig. 5.49. Host cell reaction in presence (S) or (NS) of secondary structures of *Erysiphe cruciferarum* on swede and barley



SED= +/- NS/R 7.33; S/R 2.95; NS/D 5.52; S/D 5.59
d.f.= 6

Fig. 5.59. Host cell reaction in presence (S) or (NS) of secondary structures of *Erysiphe graminis* on swede and barley



SED= +/- NS/R 12.80; S/R 1.22; NS/D 16.71; S/D 4.94
d.f.= 6

associated papillae showed no haustoria. In contrast no normal haustoria were formed in barley leaves with or without an associated papilla, thus no secondary structures were produced. Significantly more haustoria were encapsulated by barley leaves compared with swede leaves. Barley leaves also had significantly more sites without haustoria, with or without papillae, than swede leaves.

Cells responded to invasion and formation of secondary structures by the pathogen by showing no reaction or becoming degenerate. Most sites (78%) of barley leaves remained unreactive with no secondary structures formed, whereas the percentage of swede sites in this category was small (12%). The remaining barley cells became degenerate with no secondary structures. In contrast a small proportion of swede leaves had no reaction when secondary structures were present with the majority becoming degenerate in the presence of secondary structures (63%) and 17% degenerating with no secondary structures (Fig. 5.49).

Erysiphe graminis

Although germination of *Erysiphe graminis* on barley was significantly higher (87%) than swede, 81% of conidia still germinated on swede leaves (Table 5.31). Of these a small number produced only a primary germ tube. The overall appressorial germ tube length was short but slightly greater on swede (Fig. 5.51). A higher proportion of germ-tubes aborted on swede, whilst the number of normal appressoria formed was 95% of germinated conidia on barley but only 73% on swede (Table 5.31; Figs 5.50, 5.52 and 5.53). The location at which

Figs 5.50 - 5.53. Appressorial formation of *Erysiphe graminis* on leaves of swede and barley.

Fig. 5.50. Appressorial formation on swede. Stained with aniline blue (x 100 magnification).

Fig. 5.51. SEM of conidium showing primary germ tube and normal appressorium on swede leaf.

Fig. 5.52. SEM of normal appressorium on barley leaf.

Fig. 5.53. SEM of abnormal appressorium on swede leaf.

Fig. 5.50.

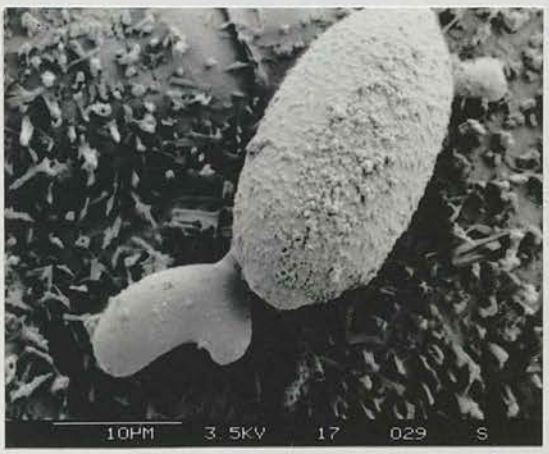


Fig. 5.51.

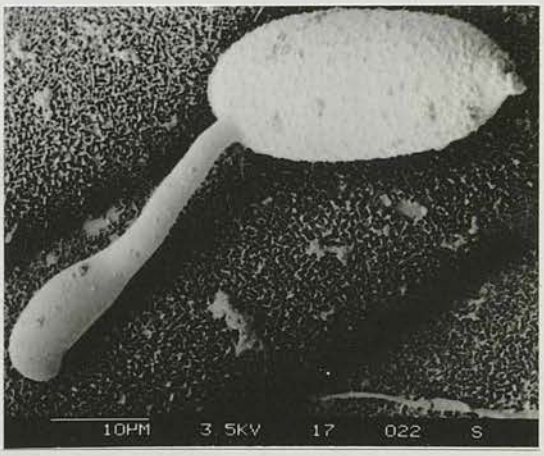


Fig 5.52.

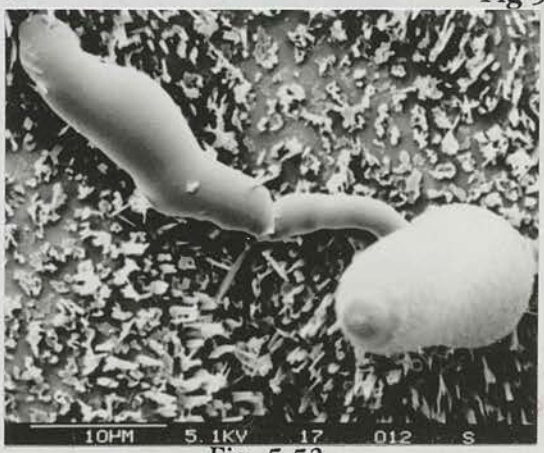


Fig. 5.53.

Fig. 5.55. Papilla formation in barley leaf in response to penetration by *Erysiphe graminis* conidium. Stained with aniline blue (x 400 magnification).

Fig. 5.56. Degenerate haustorium of *Erysiphe graminis* in epidermal cell of swede leaf. Stained with aniline blue (x 400 magnification).

Fig. 5.55

Fig. 5.56

Fig. 5.57. Development of secondary structures of *Erysiphe graminis* on leaf of barley also showing normal digitate haustorium within epidermal cell. Stained with aniline blue (x 400 magnification).

Fig. 5.60. 5.61. SEM of secondary development of *Erysiphe* graminis on barley.

Fig. 5.57.

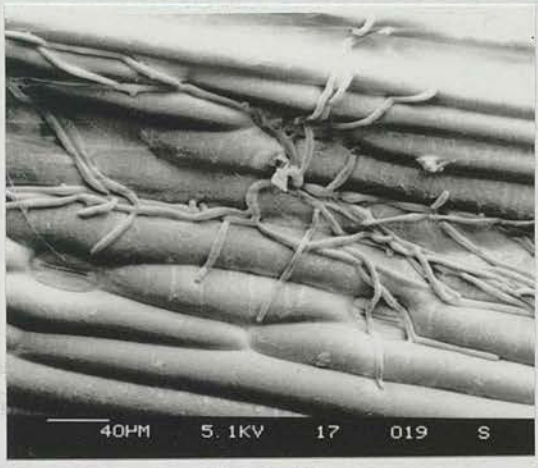


Fig. 5.60.

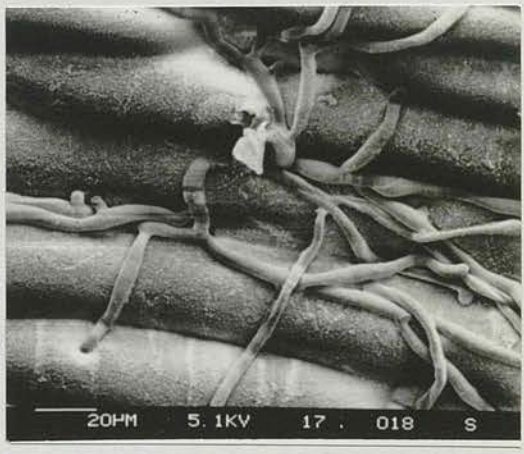


Fig.5.61.

appressoria were formed was not dependent on the host plant. On both hosts roughly half of all appressoria were established at periclinal wall areas with the other half being established at anticlinal wall areas (Fig. 5.54).

Penetration of the surfaces of barley by *E. graminis* appressoria resulted in an haustorial initial. Once again this either developed into a "normal" haustoria, always in association with a papilla, or "encapsulated" in host wall material or, in a small number of cases, became degenerate. In some instances there were papillae without haustoria and very occasionally papillae and haustoria were absent (Figs 5.55, 5,56, 5.57 and 5.58).

The major differences between the two hosts when inoculated with *Erysiphe graminis* concerned the development of normal haustoria, this being 22% in barley epidermal cells and nil in swede epidermal cells; also, no reaction without haustoria was found in 28% of swede sites and only 2% in barley sites. Slightly larger proportions of haustoria were encapsulated in barley leaves than swede leaves with similar numbers of papillae formed without an haustorium in both hosts (Fig. 5.58).

With respect to the response of the cells to invasion and formation of secondary structures by the pathogens, approximately half of swede epidermal cells did not react when no secondary structures were present, the remainder becoming degenerate without secondary structures. On barley 20% did not react when secondary structures were absent and only a small number showed no reaction when associated with secondary structures. It was seen that 33% of barley cells

degenerated in presence of secondary structures, whilst 44% degenerated when they were absent (Fig. 5.59).

Finally all haustoria which developed normally gave rise to secondary structures. Thus 20% of conidia forming appressoria successfully produced secondary growth on barley but none succeeded on swede (Table 5.31; Figs 5.57, 5.60, 5.61).

5.4 Discussion

Relatively little information is available on the pattern of germ-tube development and mode of penetration of *Alternaria* species on host plants, or responses of brassicas to invasion by the pathogens. In contrast the development of *Erysiphe cruciferarum* on crucifers is well defined (Munro, 1985). The microscopical studies of this section attempted to describe the early stages of infection by both genera in relation to visual assessment of symptom expression obtained in the previous chapter.

The three fungi represented a range of parasite specialisation (Holliday, 1989) *Alternaria alternata*, normally a saprophyte but becoming pathogenic on a wide range of plants, usually when they are weakened or damaged. *Alternaria brassicicola*, is a facultative parasite apparently specific to cruciferous plants. *Erysiphe cruciferarum* is a highly specialised obligate parasite of mainly cruciferous plants. The overall developmental process as described in the study appeared to conform to the three elementary growth strategies.

1. Leaf surface behaviour

Phases of pre-penetration growth were similar for all three pathogens in that stages of germination, germ-tube growth and differentiation into infection structures could be identified. However the *Alternaria* species formed extensive external growth for a prolonged period relative to *E. cruciferarum*; thus the potential for interaction with host surface factors was increased.

After the initial problems of deposition and retention, the germination and development of a pathogen on a leaf surface may be stimulated or inhibited by host factors (Blakeman, 1971; Wynn & Staples, 1981), but it is

generally agreed that spores of obligate parasites can germinate and advance to infection sites without any apparent assistance from their hosts (Preece, 1975) whilst facultative parasites have a certain degree of dependence on nutrient availability. Nevertheless the main factor often determining contact and initiation of infection is the availability of free surface water, governed primarily by agents regulating the wettability of the host surface.

The hydrophobic nature of the surface on the brassica canola, in the form of an even layer of fluffy wax crystals, has already been shown to reduce the rate of germination and germ-tube number of *Alternaria brassicae* (Conn & Tewari, 1989). The reasons are thought to be threefold (1) the epicuticular wax of canola creates a surface unfavourable for the retention of water-borne inoculum; (2) by restricting the volume of surface water germination is reduced and; (3) fewer germ-tubes are produced due to lack of nutrients from leachates (Conn & Tewari, 1989).

Properties similar to the surface of canola are most likely applicable to the glaucous surfaces used in the present study, and could explain observed differences in susceptibility to *A. brassicicola* from visual assessments (Chapter 4). However, no variations were seen in levels of pre-penetration development, from analyses of brassica/leaf position effects and mutant effects which would support the reasons given by Conn & Tewari (1989). It is appreciated that the method of inoculation, *i.e.* droplet inoculation, eliminated effects of deposition and retention whilst the incubation technique provided ideal conditions for germination and surface development. However, even though droplets on the surface were effectively prevented from drying, their spread, contact interface and other chemical and physical properties would still be influenced by form and density of epicuticular

waxes. Thus the degree of control leaf surface factors have on germination behaviour and early stages of infection, the main objective of the experiment, was still able to be assessed.

Evaluation of each individual stage of development (i.e. germination, germ-tube number, germ-tube length, branching of primary germ-tube, axis divergence of primary germ-tube and vesicle production) showed that behaviour of *A. brassicicola* was more or less similar on all surfaces. Any differences which did occur could not be directly assigned to obvious surface characters. Many of these variations were small, despite significant effects, and were not considered to be of any significance. The exception to this were the apical leaf effects of Brussels sprout. Consistently more growth at virtually all pre-penetration stages was observed on apical leaves of Brussels sprout compared with other brassicas and leaf positions. Experiments in Chapter 3 demonstrated these leaves had a higher affinity for water and were more permeable to ions originating in epidermal tissue. Either property could conceivably support greater vegetative growth.

The phenomenon of nutrients on the leaf surface either supporting or suppressing disease was explored in an investigation not reported here (unpublished data). Addition of inorganic salts and sucrose to inoculation droplets negated surface effects such that waxy surfaces of mutant line 90 and the wild type expressed similar levels of symptoms to the glossy surfaces of mutant line 90 and 229. In comparison hosts, challenged with sterile distilled water/spore controls revealed more severe symptoms on glossy surfaces but little symptom development on the waxy surfaces. More profuse growth was observed on the treated surfaces, thus correlations could in this case be made between amount of surface growth and degree of symptom expression (Figs 5.62 and 5.63).

Fig. 5.62. Growth of *Alternaria brassicicola* on leaf of 90WAX from inoculation droplet of sterile, distilled water (x 100 magnification).

Fig. 5.63. Growth of *Alternaria brassicicola* on leaf of 90WAX from inoculation droplet with added nutrients (x 100 magnification).

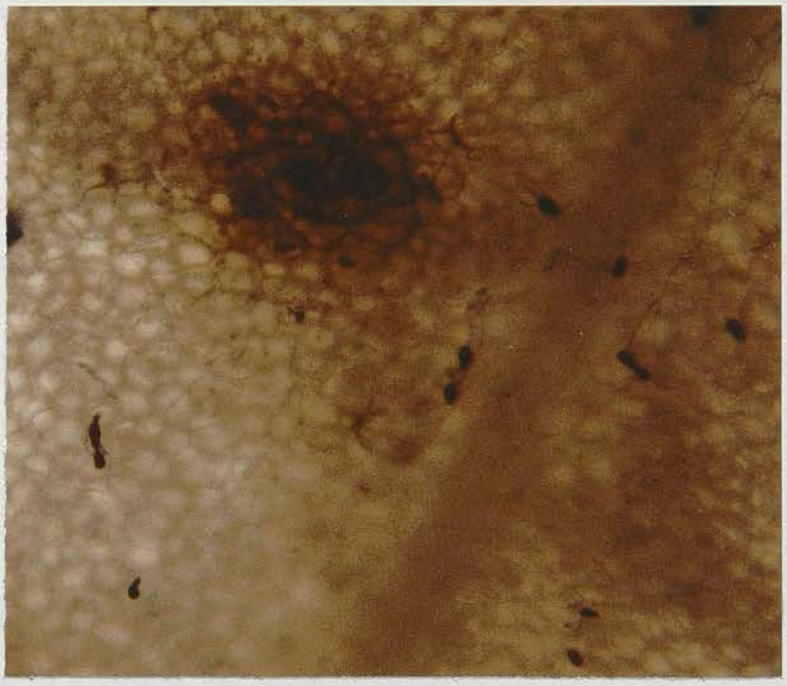


Fig. 5.62

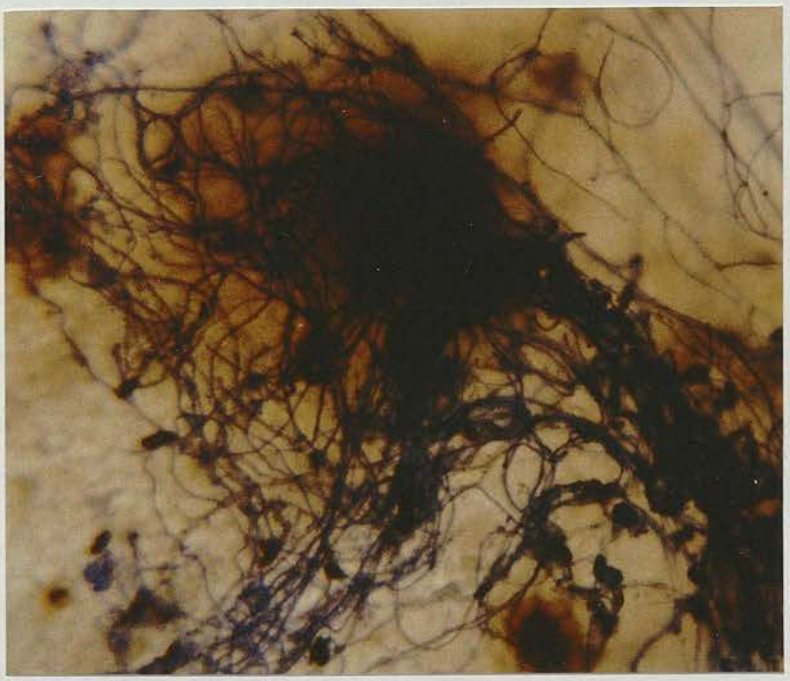


Fig. 5.63

It could be that the increase in relative permeability of the apical leaves from Brussels sprout leads to more surface mycelial development, which in turn leads to more symptom expression. However this still does not explain the permeability/symptom expression trends of other combinations especially the glossy mutant of line 229 and the basal leaves of oilseed rape (Chapters 3 and 4; Figs 3.20, 3.21, 4.4 and 4.21).

Conidia of *Alternaria* can germinate and start producing germ-tubes within six hours of initial contact with a substrate (Conn & Tewari, 1989). If leachates are to assist primary development of a pathogen there must be an immediate supply. The effects of immediate supply were shown when salts and sucrose were added to inoculation droplets. Significant changes in conductivity of droplets on surfaces (at least of mutants) due to exudation from their leaf tissues were not recorded until 10 or even 24 hours post application. By this time the pre-penetration stages of *Alternaria* would be nearing completion, and would presumably be no longer influenced by additional nutrients. Although presence of surface nutrients may account for effects on fungal growth on basal leaves, it does not adequately explain growth on the glossy mutant of line 229. Even at 0 hours appreciable levels of electrolytes were recorded in surface droplets on the mid-leaves, which by ten hours had risen to a higher level than that on the apical leaves of Brussels sprout. Yet surface development on this mutant was comparable with most other surfaces. For substantial growth, restriction within a small spherical droplet would seem undesirable even with optimum nutrient availability. Rather spread of droplets across the surface would allow maximum surface area for hyphal extension. Prasanna (1984) argued this to be the reason why the degree of *Alternaria* blackspot was less on leaves which had been inoculated in such a way that spore droplets remained intact.

Perhaps droplets on leaves of mutant 229GLO were also prevented from spreading by any surface waxes present, or by the inoculation technique. The former seems unlikely due the high wettabilities estimated for this surface in Chapter 3, whilst similar techniques were used for all surfaces but differences were still obtained. Although there was no evidence of any fungitoxic components of the wax having any role to play (Chapter 4), another explanation may be the presence of some fungitoxic or fungistatic substances co-exuded into droplets with nutrients. Such substances are widespread, occurring in many plants and are often unspecific. For example gallic acid, isolated from the leaves of *Acer platanoides* is inhibitory to *Cladosporium sphaerospermum, Cladosporium herbarum* and *Cylindrocarpon radicicola* (Dix, 1974), but occasionally is found to be produced in response to challenge by a pathogen. Diffusates from the *Phaseolis vulgaris* cv. Topcrop protected the hypocotyls from attack by *Colletotrichum lindemuthianum* (Berrand, Kúc & Williams, 1973).

Although qualitative analysis of droplets was not assessed, it is presumed they contain substances other than simple electrolytes, perhaps even microbial inhibitory or stimulatory substances. Due to the glossy nature of the 229 mutant, these substances would be expected to diffuse into droplets with relative ease and accumulate fairly rapidly, thus their effects would be accentuated. The processes of leaching and droplet spread, therefore, have important implications on pre-penetration development of splash-dispersed fungi.

Besides production of extra-cellular enzymes and toxins, exudates from spores may have implications on the leaf surface properties. Davis & Evans (1990) discovered exudates from several foliar pathogens, including *Septoria tritici* and *Pyrenopeziza brassicae* had the ability to significantly lower

contact angles of water droplets on leaves. The exudate was only effective on host leaves, thus implying the recognition process of host plants may begin before germination. SEM studies (unpublished work) revealed that inoculation droplets of *Alternaria brassicicola* on leaves of swede contain materials which precipitate or crystalise at low temperatures and appear as a matrix in which the spores are embedded. Further work proved that the materials originated from the spores and not the plant (Figs 5.64 to 5.66). Possibly an examination of this material would show it too contains a leaf wetting factor. Or it could contain enzymes for degradation of nutrients at the surface. Immediate availability of nutrients in a field situation is a possibility. *Alternaria* is more prevalent during wet weather. Being splash-dispersed the pathogen is deposited at the surface in existing droplets into which the process of leaching has probably been occurring, depending on whether the cuticle is waxy or glossy.

Pre-penetration stages of *A. alternata* were generally similar to *A. brassicicola* except that each stage of growth was generally more extensive, highlighting the saprophytic nature of this fungus. Even so, it was more difficult to derive conclusions as to what exactly was influencing the surface growth. Fewer correlations to surface properties could be made in comparison with *A. brassicicola*. This does not necessarily exclude the involvement of like forces in the interactions. Rather the responses and their expressions are subject to more variability.

Both powdery mildew fungi in this study complied with the typical character of an obligate parasite. They grew on the leaf surface for a minimum period before attempting penetration regardless of surface or indeed host.

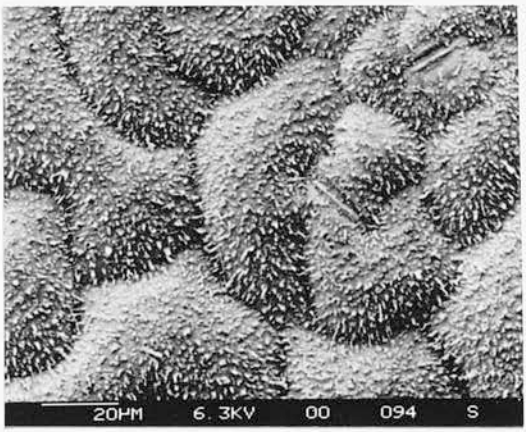


Fig. 5.64. SEM of swede leaf inoculated with sterile, distilled water droplet.

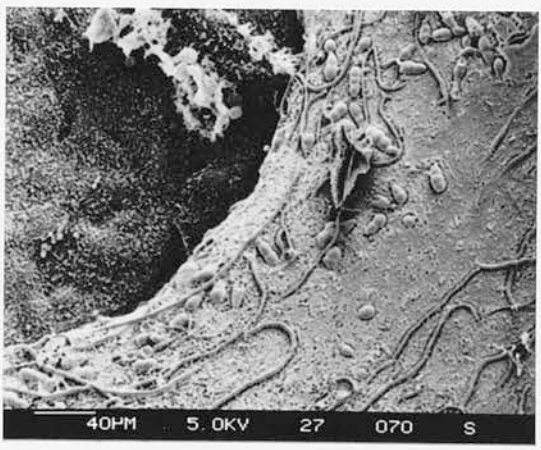


Fig. 5.65. SEM of swede leaf inoculated with "wet" spores of *Alternaria brassicicola*.

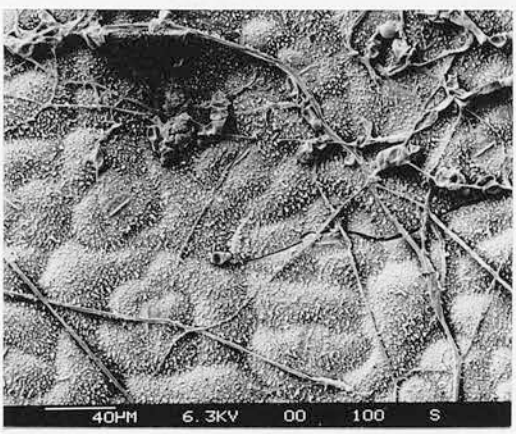


Fig. 5.66. SEM of swede leaf inoculated with "dry" spores of *Alternaria brassicicola*

Very few surface factors appeared to influence the germination of *Erysiphe cruciferarum*. Significant differences were obtained only for germination percentages in relation to *gemmifera* mutant effects and not cultivar/leaf age or host effects. Initially, germination seemed to be influenced by genotypic characters of the *gemmifera* lines, being overall higher on parental line 90. Within this line the germination rate looked to be favoured by a waxy surface. Similar conclusions were made for other lines, except for numbers of conidia germinating on the glossy mutant of line 229. Although germination is probably supported entirely by reserves there are numerous factors which may have an indirect influence. Primarily germination may depend on the conditions in which inoculum is produced. Conidia of *Erysiphe graminis* germinate with more efficiency when under controlled conditions conducive to conidial viability (Carver & Adaigbe, 1990). This tentatively explains differences in germination of *E. cruciferarum* on swede in the different experiments of this chapter. Nevertheless, physical features and properties of the phylloplane are probably the major factors determining success. Although free surface water is unfavourable to germination of *Erysiphe* species (Munro, 1985), conidia of *E. graminis* (and presumably *E. cruciferarum*) preferentially germinate in humid rather than dry environments. The wettability properties of leaf surfaces regulate the volume of free water. Glossy surfaces retain water more easily, therefore present less suitable conditions for germination. Conidia of *Erysiphe* species may require components of host plant cuticles for initiation of germination (Munro, 1985; Kunoh, Nicholson, Yosioka, Yamaoka & Kobayashi, 1990), whilst induction or inhibition by chemical means could also be expected (Singh & Singh, 1983; Carver & Thomas, 1990).

These factors help account for differences in germination but as the

results show, no single factor appears to be solely responsible for the variation. This is probably a consequence of the interaction between these and other possible factors.

Germination resulted in a short germ-tube, typical of many obligate parasites. Despite differences in germ-tube length being small, surfaces on which germ-tubes were shorter tended to be more susceptible. Analyses of germ-tube length in relation to cultivar/leaf position effects suggested it may be influenced by wettablility or permeability factors. However mutant surface analysis showed that, although within each mutant line, germ-tube length was negatively correlated with degree of waxiness, the foremost effects seemed to be from the genetic background of the mutant.

Munro (1985) proposed that surface growth may be supported by nutrients on the leaf, explaining partly the reactions seen on *gemmifera* mutants, but a more likely reason is that location of an infection site is more complicated on some surfaces than on others. The culmination of germination is appressorium formation. The induction and maturation of appressoria will be discussed later.

Comparison of *Erysiphe graminis* with *E. cruciferarum* indicated that their surface development was similar; conidia of *E. graminis* germinated, produced a short germ-tube terminating in an appressorium. The one difference was that prior to the appearance of the germ-tube destined to end in an appressorium, termed the "appressorial germ-tube", a smaller germ-tube termed the "primary germ-tube" (Carver & Adaigbe, 1990) was produced. Production of the appressorial germ-tube, primary germ-tube or both was scored as germination.

In contrast to *E. cruciferarum*, germination of *E. graminis* was slightly greater on its host compared with that on a non-host. Recent work by Nicholson et al. (1988) and Kunoh et al., (1990) has proved that on contact with a surface, conidia of *E. graminis* release an esterase, perhaps a cutinase, which degrades wax components of barley cuticles. It was proposed that the cuticular erosion either reveals stimuli on the surface ensuring correct timing of morphogenesis, or prepares the infection court (Kunoh et al., 1990) or simply provides a substrate for the process of penetration (Nicholson et al., 1988). Whatever the function it would appear the enzyme is not for the purposes of recognition and specificity; 81% of conidia of *E. graminis* germinated on swede leaves and 83% of these produced appressoria. Whilst both figures were significantly lower than those on barley leaves, they are still reasonably high.

On assumption that the esterase is secreted on contact with both surfaces and that swede and barley waxes have individual chemistries, it is possible that even though the enzyme is capable of degrading both waxes, the rate of degradation is different. Thus the exposure of stimuli or release of degradation products would occur more slowly on swede than on barley and, in turn, the processes of development dependent on these would be delayed.

Production of an appressorial germ-tube is thought to be conditional on contact of the primary germ-tube with host cells (Carver & Adaigbe, 1990). Either organic and inorganic salts are taken up through the primary germ-tube to supplement the appressorial germ-tube or more simply that access to host water is required for further growth (Carver & Adaigbe, 1990). The workers concluded that some resistance in barley cultivars and adult plant resistance was probably due to thicker cuticles preventing the primary germ-tube from successfully accessing host tissues, thus impeding germling

development. However, they do not mention which stratum of the cuticle may be responsible for this resistance.

The results of this study suggest that the primary germ-tube of *E. graminis* is also not involved in recognition of a suitable host. Providing contact was made with host cells, either on swede or barley, an appressorial germ-tube was produced, hence both germ-tubeswere produced in almost all cases on both hosts. If, as Carver & Adaigbe (1990) imply, features of the cuticle are the primary factors of germ-tube/host association, and if barley and swede surface waxes have a contrasting morphology as indicated by SEM observations, yet both support high numbers of appressoria, it might be argued that configurational properties of the wax are not important. Thickness of the cuticle proper (Fig 3.1) is probably more important.

Penetration by the primary germ-tube was seen to occur on many occasions on swede, indicated by a fluorescent reaction at the sight of penetration. The primary germ-tube may therefore have a similar role on swede leaves as that proposed by Carver & Adaigbe (1990) for barley leaves but this remains unconfirmed.

Penetration.

For those pathogens which gain entry into host tissues by breaching intact surfaces the penetration process begins with formation of infection structures. Infection structures encompasses hyphae which penetrate surfaces without any apparent prior morphogenesis, for example the hyphal mass of infection cushions formed by *Rhizoctonia solani*, and more commonly appressoria (Dickenson & Lucas, 1977). Appressoria are defined as "swellings formed at the end of a germ-tube and have the capacity to adhere to the plant surface and the ability to effect penetration" (Dodman, 1979). Form and function of appressoria are defined for many plant pathogens but the mechanisms of penetration are less well understood (Dodman, 1979).

The vesicles formed by both *Alternaria* species seemed to satisfy the appressorial description. They were formed singly, mostly as terminations of hyphal growth, and in preliminary observations were seen to initiate localised host reactions directly below the structures, even if intercalary (see Fig. 5.5).

A feature, especially *A. alternata*, of *Alternaria* appressoria was that on some occasions more than one was formed on the germ-tube, hence some were intercalary as well as terminal. The intercalary appressoria probably represented failed penetrations, the germ-tube growing on and attempting a further penetration from a second appressorium. The phenomenon of successive penetration attempts is comparable with the mode of infection by *Erysiphe graminis*, where multiple lobed appressoria are produced. Each lobe is the origin of a penetration peg (Carver, 1986).

Nevertheless, despite the presence of two or sometimes three appressoria on some germ-tubes, when analysed overall, usually only one was produced from each *A. brassicicola* germ-tube regardless of surface type.

Once again, any differences obtained were minimal. Termination of hyphae in appressoria occurred on all surfaces at a high percentage except on the waxy phenotype of line 90 and the apical leaves of Brussels sprout.

Percentages of *A. alternata* germ-tubes terminating in a vesicle were overall smaller than seen for *A. brassicicola*. Comparing the two *Alternaria* species in relation to appressorial formation, the more specialised pathogen, i.e. *A. brassicicola*, seems to be more efficient in forming infection structures. Appressorial production by *A. alternata* was slightly more variable than that of *A. brassicicola*, but some correlation between waxiness and vesicle (appressorial) numbers was observed.

Induction of appressoria is an intriguing subject. Mechanisms involved in the response are poorly understood but are known to be mediated through either chemical or physical stimuli or both. Parberry & Blakeman (1978) suggest that whilst nutrient stress alone can be a major factor in appressorial induction in *Colletotrichum acutatum*, in other pathogens nutrients are required (Wynn & Staples, 1981). Other chemical factors associated with the leaf surface may also be involved. In addition to stimulating germination, the leachates of banana and apple enhance appressorial formation in *Colletotrichum musae* and *Diaporthe perniciosa* (Brown & Swinburne, 1978).

Most of the directly penetrating fungi form appressoria in response to a contact stimulus, usually a hard substrate which allows adherence (Wynn & Staples, 1981). Some form appressoria at random over the cuticle surface but more often specific sites are selected. At least 22 species in 14 genera form appressoria at epidermal cell junctions (Wynn & Staples, 1981). These include *Peronospora* (Preece, Barnes & Bailey, 1967), *Colletotrichum* (Lapp &

Skoropad, 1978) and *Septoria* (Baker & Smith, 1978). Termination of germ-tube growth occurs at the cell junctions for three possible reasons: (1) the crevices created by the topography of the epidermal cells often accumulates nutrients; (2) the cuticle in these areas is diminished (Kirkwood, 1972); (3) the incline of the adjacent cell represents a line of resistance causing germ-tubes to swell.

Alternaria brassicicola showed a high specificity towards termination of hyphal growth at the anticlinal wall and, as discussed earlier, there formed an appressorium. The exception to this were middle leaves of oilseed rape and apical leaves of Brussels sprout. The latter were also unusual with respect to vesicle number and percentage of germ-tubes terminating in a vesicle. The low specificity for anticlinal walls of oilseed rape is more confusing. Perhaps the thickness of the epicuticular wax on this brassica obscures surface topography, such that anticlinal walls cannot be detected. Although *A. alternata* overall tended to form appressoria at anticlinal walls, the selection of sites seemed to be more variable.

It was difficult to assign gross surface features as stimuli for appressorial induction. Appressorial induction in *Alternaria* appears to be contact stimulated, but may be modified by other factors. The presence of nutrients on the apical leaves of Brussels sprout may explain the unusual observations seen for *A. brassicicola* on these leaves. Factors such as surface topography, epicuticular wax form or epicuticular wax chemistry may be important in determining where appressoria form. The high specificity shown by *A. brassicicola* for anticlinal wall areas regardless of cultivar, leaf position or wax type suggests that the stimulus must be common to all surfaces. *Alternaria alternata* appeared less discriminating with respect to site of penetration, possibly in keeping with its unspecialised facultative parasitism.

Appressorial induction in *E. cruciferarum* took place efficiently on almost all brassica surfaces and on the non-brassica surface. The only exception to this was the effects seen with *gemmifera* mutants, where germ-tubes aborted before transformation into appressoria more frequently on those surfaces which were overall more resistant. Appressorium formation in *Erysiphe* species is believed to be part of a "pre-programmed" developmental process, nevertheless certain characteristics of plant surfaces are thought to be stimulatory to their induction (Munro, 1985; Nicholson *et al.*,1988). Thus high numbers of abortive germ-tubes are possibly due to lack of this cuticular component, either because of phenotypic or genotypic variation. It may be noted, however, that numbers of appressoria were relatively high on the glossy mutant of line 229, which was fairly resistant to mildew. In this case the number of abortive appressoria was high. Numbers of appressoria produced by *E. cruciferarum* on barley leaves, a completely unrelated surface both genetically and phenotypically, were similar to those on the swede cultivar Doon major. This observation again conflicts with the idea of a host stimulus to appressorial formation. It is not possible to say what is responsible for appressorial formation in *E. cruciferarum* at this stage. The subject merits further investigation, and perhaps once the stimulus is known it could be manipulated to suppress formation of infection structures.

The size of *E. cruciferarum* appressoria on brassica leaf surfaces offered some impression of the extent to which they matured. If compatible surfaces were conducive to appressoria of optimum size then on incompatible surfaces appressoria either formed abnormally or were relatively large. This was also found in the case of *E. graminis*.

Based on their observations that conidia of *E. graminis* formed few normal appearing appressoria on the lower surface of epidermal strips, the

lower surface of isolated cuticles and plants with the eceriferum (a wax) mutation, Yang & Ellingboe (1972) concluded that formation of mature appressoria depended on properties of the host surface especially the wax layer. However ungerminated conidia of *E. graminis* have since been shown to release an esterase which degrades the epicuticular waxes (Nicholson *et al.*, 1988). According to these authors the waxes required for successful appressorium formation would no longer be intact at this point. They believed some other component of the cuticle to be crucial. More recent work by Carver & Thomas (1990) demonstrated that germlings of *E. graminis* developed normally on intact oat leaves and wax-free leaves. They also concluded that the physical structure of epicuticular waxes was not involved in the recognition processes leading to normal appressorial development.

Factors controlling appressorial formation in *Erysiphe* may not be directly related to the configuration of the epicuticular wax. Results of this study suggest that some wax element, however, has important influence. Carver & Thomas (1990) speculated that whilst physical properties are not involved with germling development, chemical signals could be the principal initiators. Perhaps degradation products are necessary precursors. Wolkow, Sisler & Vigil (1983) have shown that if melanin synthesis is inhibited in *Colletotrichum lindemuthianum* the appressoria are misshapen and cannot penetrate intact surfaces. Conceivably precursors may be used to bestow hardiness properties on the appressoria. Alternatively, the exposed areas of the cuticle or remnants of degraded waxes may reveal attachment points to which the appressoria adhere. Differences in sizes of appressoria may be an indicator of their prevalence.

In contrast to *A. brassicicola*, *E. cruciferarum* preferred the periclinal wall area for appressorial formation. Since the purpose of penetration of *E.*

cruiferarum is to establish a direct link with host epidermal cells with minimum delay, then entry through the most straightforward route is preferable. *Erysiphe cruciferarum* has a more highly adaptive apparatus for penetration than *Alternaria*, thus is able to overcome the additional obstruction presented by the periclinal wall. Factors inducing appressoria to form at periclinal walls are difficult to identify, but are possibly gross topographical features relating to the profile of the epidermal cells. Major differences were observed between swede and barley leaves.

Erysiphe graminis, is considered to form appressoria indiscriminately on the leaf surface (Wynn & Staples, 1981). In this study, *E. graminis* seemed to favour equally anticlinal and periclinal walls. Russo & Bushnell (1989) have found that even though these appressoria may appear to form over the anticlinal wall, the actual point of attack is periclinal, about 4 μm from the cell junction. Thus appressoria which formed over the anticlinal wall in this study, and were presumed to penetrate the anticlinal wall, probably were attempting to breach periclinal barriers. As previously mentioned this is the more obvious route for obligate *Erysiphe* species.

For directly penetrating fungi, the penetrative hypha, otherwise known as the "peg", forms at the centre of the area where the appressorium adheres to the cuticle or to an inert surface (Wynn & Staples, 1981). Popular opinion on the mechanism of penetration states that ingress of the penetration peg is at least assisted by enzymic activity (Dodman, 1979). Most commonly the presence of cutinase is quoted as a prerequisite for penetration of intact surfaces.

Cutinaseless mutants of *Colletotrichum gloeosporioides* are unable to effect penetration of papaya (Dickman, Patil & Kolatukudy, 1982). Cutinase

has been shown to be a determining factor for infection by *A. alternata* (Tanabe, Nishimura & Kohmoto, 1988). Further research where a range of cutinases from unrelated pathogens were assessed demonstrated a specificity not for host but for tissue (Trail & Köller, 1990). For example amendment of *Rhizoctonia solani* with cutinase from *Venturia inaequalis*, a leaf infecting pathogen, enabled *R. solani* to similarly attack leaves whereas only stems were previously infected and *vice versa*. *Alternaria brassicicola*, a pathogen of both stems and leaves possessed both types of cutinases.

Features of the cuticle proper were important in determining the degree of infection by both *A. brassicicola* and *A. alternata* based on the correlations obtained for numbers of penetrations leading to sub-cuticular hyphae and degree of symptom expression. Thickened or hardened cuticles are considered to be significant resistance elements in many plant-pathogen combinations (for example Marks, Berbee & Riker, 1965; Castledine, Grant & Roberts, 1981). Thickness of the cuticle proper may explain the differences in susceptibility of waxy and glossy surfaces to penetration. However, the degree of waxiness is not always an indicator of cuticle thickness (Martin & Juniper, 1970). Since cuticle thickness was not measured, the possibility that the cuticle presents a physical barrier is only conjecture.

Nevertheless if cuticle thickness is a major factor and, as Trail & Köller (1990) suggest, *A. brassicicola* possesses cutinase capable of unspecific degradation of host cutin, why are such differences evident? Cutinase supposedly is secreted at a constant rate and not in proportions matching the volume of substrate present. Thus on cuticles that are relatively thick insufficient enzyme would be secreted for complete breaching. On the other hand if differences in cuticular thickness turned out to be negligible, then it is

proposed waxes on the surface are preventing contact with the cuticle proper in the same manner described in Chapter 3.4 for wettability.

The latter would also explain the success of some penetrating hyphae on relatively resistant surfaces. As stated in Chapter 3, the density and configuration of waxes would be significant in determining the droplet-surface interface. The only surfaces which did not conform to this theory were apical leaves, especially those of swede. An additional point, also discussed in Chapter 3, was the fungitoxic nature of the ketone fraction of brassica wax. The release of ketones through enzymic degradation was not considered particularly significant due to ketones being at a constant level for all leaf positions. The presence of some other fungitoxic factor cannot be dismissed. Perhaps it is not the ketone fraction but the relatively high levels of esters in swede apical leaves that is the toxic moiety. If this is the case then it seems the toxicity is specific to *A. brassicicola*.

The involvement of the cuticle in resistance to penetration by both *Erysiphe* species was indicated by presence or absence of a papilla in the underlying epidermal cells. Overall at least 70% of *E. cruciferarum* appressoria gained successful entry through the cuticle of the various hosts examined. However of those that were stopped the nature of the cuticle was apparently decisive in preventing host cell contact. Thus all surfaces of mutant line 90 were penetrated to a higher degree than surfaces of line 229. Swede leaves were relatively more vulnerable than barley leaves to *E. cruciferarum* but less vulnerable to penetration by *E. graminis*.

Penetration by *E. graminis* may involve chemical plus physical factors (Kunoh *et al.*, 1990). If the same can be inferred for *E. cruciferarum*, any differences in cuticular penetration is possibly due to deficiencies in

substrates liberated through enzymic activity. Nicholson *et al.* (1988) have proposed such substrates have nutritive value in the penetration process.

With respect to leaf position only the intact surfaces of Brussels sprout leaves showed any degree of cuticular resistance. The apical leaves of swede were also, interestingly, less favourable to ingress by *E. cruciferarum* in much the manner as *A. brassicicola*. This lends support to the previous supposition that these leaves have an inhibitory component in their waxes or cuticle. The toxic factor seemed to be restricted in its activity towards fungi which are pathogenic to brassicas.

Early infection events.

Having overcome the initial barrier of the cuticle, the species from both genera of pathogens assumed different lifestyles.

A. brassicicola and *A. alternata* became sub-cuticular, forming in some cases complex networks of sub-cuticular hyphae. The extent of sub-cuticular growth often determined the size of the necrotic lesions. In contrast *E. cruciferarum* and *E. graminis* directly penetrated the epidermal cells and formed haustoria. If a compatible relationship was established with the host surface secondary structures were formed.

Several pathogens develop sub-cuticular hyphae after penetration. Most information concerns those pathogens which infect fruits. The sub-cuticular phase is thought to extend the facultative stage until the underlying tissue ripens and can be readily parasitised (Daykin & Milholland, 1984; Tewari, 1986). Tewari (1986) does not believe latency of infection to be the reason why *Alternaria brassicae* forms sub-cuticular hyphae, rather the temporary stage enables secretion of enzymes and toxins which facilitate conditioning of

the epidermal tissue for parasitism. Since *A. brassicicola* is closely related to *A. brassicae* it is possible that a similar mechanism applies.

An unusual feature of *A. brassicicola* and *A. alternata* sub-cuticular hyphae were the distortions, which appeared either proximal or distal to the penetration point. On mutants they were least frequent on glossy leaves but when brassica/leaf position effects were assessed, the opposite was observed. They could not be linked to any one factor, nor could it be said if they are advantageous or disadvantageous effects for the pathogen. Whether they arose due to stress or whether they have a specific function is not known. Therefore further work to explain the phenomenon is suggested.

Sub-cuticular hyphae were typically seen to follow the lines of cell junction, possibly because these offer less resistance. In addition degraded pectin from the middle lamella and cuticular membrane forms a ready substrate for hyphal extension, assisting rapid ramification of hyphae and increasing the area of colonisation.

Host cells were seen to react to ramifying sub-cuticular hyphae by forming fluorescent plugs, usually at the corners of the cell junctions. The papillae were considered to be points at which the sub-cuticular hyphae encroach the epidermal wall before being diverted along the adjacent cell junction. Analysis of host reaction in relation to penetration and invasion of a pathogen which has a sub-cuticular mode of growth is complicated. Normally papillae are produced in response to a wound, be it either the result of mechanical disruption or pathogen invasion (Aist, 1976). They prevent leakage of metabolites from the cell or present a barrier to an attacking structure to gaining entry. The cuticle can then be thought of as having a further role in determining the speed of pathogen ingress and in turn the

point and time of host defence initiation.

In relationships where the pathogen becomes sub-cuticular, direct penetration of the pathogen into epidermal tissue is possibly insignificant at least during early invasion progress. Even though papillae may prevent initial cell penetration of the pathogen, the eventual colonisation would be unaffected. Necrotic reactions of host epidermal cells in association with sub-cuticular hyphae were deemed to be more important indices of the progress of infection. Indeed correlations were observed between susceptibility of tissue, number of sub-cuticular hyphae and numbers of associated necrotic areas.

A fairly new concept in resistance of brassicas to *Alternaria brassicae* is the production of phytoalexins (Conn, Tewari & Dahiya, 1988). Phytoalexin production is perhaps part of the hypersensitive response. Therefore the absence of necrotic cells in incompatible combinations may be twofold. Reduced numbers of sub-cuticular hyphae are due to impedance by the epicuticular wax and/or cuticle, or cellular resistance from phytoalexin production and hypersensitivity products. It is worth mentioning here that hypersensitivity is different from general necrosis. Necrosis was considered to be general cell death from the effects of either pathogen toxins or enzymes. Hypersensitivity which was microscopically differentiated from necrosis by appearing as bright fluorescence, is the induction of the cell defences which culminates in death. Such mechanisms may be the reason why cells from mutant line 99GLO and the apical leaves of Brussels sprout have unusually low numbers of necrotic cells.

Munro (1985) associated differences in susceptibility of various brassica cultivars to *E. cruciferarum* with restriction of colony development beyond

the appressorial stage. He concluded host resistance to mildew may be expressed as reduced mycelial growth and spore production. In the present study conidia of *Erysiphe cruciferarum* were seen to have the potential of forming six germ-tubes from each conidium, with branches and conidial initials from each of these germ-tubes. Generally if conidia were successful in forming secondary structures, at least the main four were produced. Differences which arose on the various surfaces, and this could be correlated to susceptibility, were mostly at the X and Y germ-tube positions. Thus, similar conclusions can be drawn from observations of colony extension in the present study to those made by Munro (1985), confirming the quantitative nature of resistance of brassicas to powdery mildew, indicated by the variations in numbers of secondary structures. Hence apical leaves were less favourable to colony initiation along with mutant line 229, 99GLO and the wild type.

The previous section discussed the importance of the cuticle in determining success of infection and concluded that it had a limited role. Probably the crucial point in the relationship is the differentiation of the penetration peg into a mature, functional haustorium. In other words the compatibility systems are located at the tissue level. Colony development of powdery mildew fungi relies on an efficient haustorium at the host plasmalemma interface for adequate nutrient supply. If efficiency of this haustorium is restricted in any way that impairs nutrient transfer from host to pathogen, then presumably secondary development is retarded. Therefore, conidial initials were observed frequently only on swede, a known susceptible host which probably allows greatest haustorial maturation, whilst the colony developed to the germ-tube stage only on surfaces of 229GLO implying reduced haustorial development.

Haustoria of *E. graminis* commence as a central haustorial body from which digitate processes elongate (Carver & Carr, 1978). Restriction of haustorial development in some cultivars of oat was found to reduce proportionately colony expansion (Carver & Carr, 1978). On a few occasions in this study *E. graminis* was observed to form a haustorial body in swede epidermal cells without development of digitations. No secondary structures arose as a result. This provides a complete range of compatibility based on functional haustoria, again implying resistance factors are mainly physiological.

An analogous system perhaps exists for crucifer mildew. Haustoria of *E. cruciferarum* are more globose than *E. graminis*, but nevertheless appear to have a network of protrusions. The relief of these structures was probably best illustrated by the wall based encapsulations of barley cells, when invaded by *E. cruciferarum* penetration pegs. The wall depositions were circular with spinelike projections. As already stated, "papillae may assume the shape of the invading moeity and thus form an encasement" (Aist, 1976). This also implies wall depositions in barley are effective elements of resistance, as on no occasion was any fungal structure seen to grow through the papillae. The work of Russo & Bushnell (1989) supports this idea. They found encasements produced in barley leaves in response to puncture by microneedles differed in secondary composition to those in response to attempted penetrations by *E. graminis*. The former contained cellulose and pectin whereas the fungal induced papillae contained phenols and basic staining material, components which have been associated with effective prevention of penetration (Aist, Gold, Bayles, Morrison, Chandra & Israel, 1988).

Munro (1985) found similar mechanisms existing in brassica cultivars in response to penetration by *E. cruciferarum*. He discussed the importance of

callose deposition in some cases of host resistance, drawing attention to the higher percentage of haustoria being encapsulated with callose on more resistant hosts. The restriction of molecular transfer from host to haustoria was suggested to be the primary function of callose.

As shown by Munro (1985) on most hosts, numbers of papilla correlated with number of secondary structures, implying they are produced in response to disruption by the penetration structure. Bird & Ride (1981) have similarly associated numbers of papillae which were lignified with extent of fungal colonisation and not degree of resistance. The survey on host structures did not assess the size of papillae (thought to be of significance by Munro, 1985), their composition or the degree of hypersensitivity. It is proposed that a further more comprehensive study be carried out to examine host cell reactions.

Even though papillae may not hold the central role in cellular resistance they are still considered to be components of the host multiple defence systems and may be linked and proceed from the same recognition events (Ride, 1983). It would be reasonable to assume that these would act in a complementary manner. Papillae may restrict fungal penetration until levels of antifungal compounds reach effective concentrations. Conversely antifungal compounds may restrict fungal growth until completion of papillae formation.

CHAPTER 6

GENERAL CONCLUSIONS

6. GENERAL CONCLUSIONS

It has long been recognised that the activities of pathogens at the host surface prior to possible invasion, in part determines a plants susceptibility or resistance to disease. An understanding of these interactions could lead to the development of techniques which interfere with the process of infection and help protect the plant from fungal attack. For this reason this study took a novel form of approach in that the impact of the host plant surface on fungal pathogens with different lifestyles was explored.

Characterisation of the epicuticular waxes over a wide range of differing brassica surfaces indicated that chemical and physical factors were responsible for their configuration. Waxes from all surfaces, including those of different cultivars, leaf positions and mutants were based on eight analogous fractions, but in different proportions. Significant amounts of hydrocarbons, secondary alcohols and ketones coincided with the appearance of crystalline conformations which bestowed glaucousness. Ontogenic changes in hydrocarbons were believed to be partly responsible for loss of crystals on basal leaves.

Genetic mutations in parental lines of *Brassica oleracea* var. *gemmifera* caused the most dramatic changes in crystal morphology. Two particular aberrations were identified. One in which conversion of C_{30} acyl groups to C_{30} alkanes was impaired, and a mutation not previously mentioned in the literature where conversion of biosynthetic system two intermediates into system one fatty acids was ineffectual. Apart from inherent factors, physical forces influenced crystalline structure. Temperature and light intensity modified size, density and configuration of crystals.

Differences in the chemical and physical properties of the epicuticular wax layer were related to differences in wettability, permeability and fungitoxicity. Water repellancy correlated with crystal type and not wax amount, and was greatest when the waxes crystallised in tubes or rods. However it was found that crystal morphology alone did not explain wettabilities. Densities of crystals were assumed to be equally significant. Freeze-fracturing did not give a reliable indication of surface cover, thus application of SEM techniques which reveal topographical views would either confirm or refute the matter. In addition SEM studies are suggested in order that modifications of waxes, if any, by surfactants be examined.

Although the precise effects by epicuticular waxes on permeabilities were not established by examining conductivity of droplets on leaves from different brassica cultivars, different leaf positions, and different *gemmifera* mutants, assessments suggested that at least in brassicas, wax configurations have important implications in understanding mechanisms of surface leakage and possible pathogen response.

Bioassays of fractionated waxes showed that the ketone fraction had fungitoxic activity. However, variation in ketone levels did not seem to account for differences in susceptibility of different leaves to infection by *Alternaria* or *Erysiphe*.

Visual assessments on disease expression showed a relationship between infection by both *Alternaria* pathogens and wettability of the leaf surface. Factors which increased wettability were also seen to increase levels of blackspot. The magnitude of the reaction however was dependent on the initial glaucousness of the surface. Thus apical and basal leaves which were least water repellant were least modified by other factors in terms of

wettability and disease expression. Although the present study did not find wettability and permeability properties of host surfaces influenced the surface behaviour of *Alternaria* to any degree, the effects of surfaces on spore deposition were not considered and requires further study.

In contrast susceptibility of *Erysiphe cruciferarum* infection seemed to be determined by the genotypic background of the host rather than the phenotypic characters. With respect to leaf position, mid-leaves, and in some instances basal leaves, were more susceptible; apical leaves expressed reduced symptoms along with basal leaves when they were believed to be undergoing senescence. More active physiological resistance mechanisms were thought to explain in part the lower susceptibility of younger leaves. On older, basal leaves, low infection levels may be linked to the failure of dying cells to meet the nutritional requirements of a biotroph.

Microscopic analysis of leaf surface behaviour of the three test pathogens plus *Erysiphe graminis* showed that the surface features examined had little influence on pre-penetration growth which could be related to the level of infection eventually attained. This is not to say that pre-penetration growth is unaffected by surface factors. Moreover, many potential forces were not tested. For example qualitative and quantitative analysis of leachates may reveal the presence of either essential nutrients, fungitoxic or stimulatory substances. Further studies to determine their prevalence and consequences on surface development are recommended.

All four pathogens were found to penetrate the cuticle surface from appressoria. The known brassica pathogens selected distinct sites for appressorial formation; *A. brassicicola* favoured anticlinal terminations, whilst *E. cruciferarum* preferred periclinal walls. The two sites were

considered to be most appropriate locations with regards to the pathogens lifestyles. Although not clear from microscopical observations, *E. graminis* was also thought to penetrate periclinally. The weak pathogen *A. alternata* was less selective and was assumed to be less efficient in detecting surface stimuli.

Maturation, if not formation of appressoria by *Erysiphe* pathogens was affected by the type of surface. The actual stimuli responsible for maturation were unknown, but were deemed not to be configurations of epicuticular waxes. They could have been either stimuli exposed after wax degradation or chemical signals released from the process. However this is unconfirmed and requires further deliberation.

After penetration *Alternaria* was found to have a sub-cuticular phase. This was thought to be a mechanism for conditioning the underlying tissue for parasitism. Success of penetration was depicted as presence of sub-cuticular hyphae in some cases hyphae showed a distorted growth, the nature or function of which is unknown. Numbers of successful penetrations by *Alternaria* were correlated with visual assessments of blackspotting. Due to lack of measurements of cuticle thickness, it was unknown whether lower susceptibility to disease was from inability to breach barriers of greater dimensions or whether higher densities and configurations of wax crystals prevented contact of water droplets, containing spores, with the cuticle proper. Future SEM studies would resolve this issue. Neither cuticle thickness nor wettability were believed to explain the curiously low levels of disease and sub-cuticular hyphae in the apical leaves of swede. Additional factors were thought to be involved, perhaps a fungitoxic element of the wax. In any case it seems the degree of infection is fundamentally determined by the

numbers of successful cuticular penetrations.

Analysis of successful penetrations by *E. cruciferarum* by microscopic observation supported visual assessments, in that establishment within the host seemed to be primarily dependent on the physiological nature of the plant cells with little regard to surface waxiness. Events at the fungal cell-plant cell interface seemed to be crucial in explaining differences seen on the surface. The production of papillae seemed a general rather than resistance response to cell assault by *Erysiphe*, but sometimes haustoria were encapsulated in callose when infection had failed. Cell degeneration, which could be interpreted as a hypersensitivity response, could also be linked with infection failure.

The overall conclusions of the study therefore are that epicuticular waxes play a significant role in dictating the properties of brassica surfaces. This in turn can have important implications to fungi on the surface which have the potential to become pathogenic, especially those which are splash-dispersed. Differences in leaf surface characteristics are reflected in only small differences in surface behaviour of *Alternaria* species, yet are still associated with significant differences in levels of disease they cause and suggest the importance of further studies on the penetration behaviour of these pathogens. Passive defence factors at the leaf surface are of minor importance in interactions with obligate parasites such as *Erysiphe* species. Rather the recognition factors determining compatibility are found in the tissues.

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APPENDICES

APPENDIX 2.1. Formulation of sphagnum moss peat compost.

1 bale (340 l) Sphagnum moss peat

140g Fritt (trace elements)

140g Potassium nitrate

140g Ammonium nitrate (Nitram)

500g Superphosphate

760g Ground limestone

760g Dolomitic limestone

APPENDIX 3.1. Methods for identification of epicuticular wax fractions. Tests used for each fraction in order of elution on P.L.C. plate.

1. Fatty acids- Identified by comparison with commercial standard, lignoceric acid (Sigma, Poole).

2. Ketols- Comparison of R.F. values with known R.F. values.

3. Primary Alcohols- Identified by comparison with commercial standard, hexacosanol (Sigma, Poole).

4. Secondary alcohols- A freshly prepared 3% ethanolic solution of vanillin with 0.5 ml concentrated H_2SO_4 added sprayed onto plates. After spraying plates were heated at 120 ^{o}C until blue spots appeared (Holloway & Challen, 1966).

5. Aldehydes- A 0.5% ethanolic solution of 2,4-dinitrophenylhydrazine with 1 ml 25% HCL added was sprayed onto plates. Carbonyl groups gave yellow derivatives, detected as yellow spots (Holloway & Challen, 1966).

6. Ketones- Positive 2,4-DNPH test; Navy-blue spots with vanillin-H_2SO_4.

7. Esters- Plates were treated with 2 *N* methanolic KOH. Esters were saponified and disappeared from plate (Holloway & Challen, 1966).

8. Hydrocarbons- Identified by comparison with commercial standard n-nonacosane (Sigma, Poole).

APPENDIX 3.2. Abbreviated ANOVA of results from Experiment 3.3.4b. Concentration of surfactant (%), required to wet leaves of three brassica cultivars (cv), in relation to treatment with commercial surfactant Agral (sf) and leaf position (lp).

Source of Variation	df (mv)	ms	VR	sig
sf	1	5.3×10^{-3}	68.7	***
residual	4	7.8×10^{-5}		
cv	2	2.0×10^{-3}	20.6	***
sf.cv	2	1.3×10^{-4}	1.3	ns
residual	16	9.9×10^{5}		
lp	5	5.7×10^{-3}	31.7	***
sf.lp	5	8.5×10^{4}	4.7	***
cv.lp	10	4.4×10^{-4}	2.4	*
sf.cv.lp	10	2.7×10^{-4}	1.5	ns
residual	116 (4)	1.8×10^{-4}		

df - degrees of freedom

mv - missing values

ms - mean squares

vr - variance ratio

sig - significance

* - significant at p = 0.05

** - significant at p = 0.01

*** - significant at p = 0.001

APPENDIX 3.3. Abbreviated ANOVA of results from Experiment 3.3.4c. Concentration of surfactant (%), required to wet leaves of three brassicas (cv) grown in different temperatures (temp) and light intensities (liin) and in relation to leaf position (lp).

Source of Variation	df (mv)		ms	VR	sig
temp	1		8.6×10^{-3}	52.5	**
residual	4		1.6×10^{-4}		
liin	1		1.6×10^{-3}	22.6	**
temp.liin	1		4.8×10^{-4}	6.9	*
residual	7	(1)	7.1×10^{-5}		
cv	2		4.0×10^{-3}	66.9	***
temp.cv	2		9.4×10^{-4}	2.6	***
liin.cv	2		2.6×10^{-5}	0.4	ns
temp.liin.cv	2		9.5×10^{-5}	1.6	ns
residual	30	(1)	6.0×10^{-5}		
lp	5		9.7×10^{-3}	156.8	***
temp.lp	5		4.5×10^{-4}	7.5	***
liin.lp	5		3.4×10^{-4}	5.4	***
cv.lp	10		8.8×10^{-4}	14.2	***
temp.liin.lp	5		6.0×10^{-5}	1.0	ns
temp.cv.lp	10		3.9×10^{-4}	6.2	***
liin.cv.lp	10		1.8×10^{-4}	2.9	**
temp.liin.cv.lp	10		2.1×10^{-4}	3.4	***
residual	224	(16)	6.2×10^{-5}		

APPENDIX 3.4. Abbreviated ANOVA of results from Experiment 3.3.4d. Concentration of surfactant (%), required to wet leaves of gemmifera mutants (ml) in relation to leaf position (lp).

Source of Variation	df (mv)	ms	VR	sig
ml	7	9.2×10^{-3}	92.0	***
residual	28	1.0×10^{-4}		
lp	5	4.6×10^{-3}	31.3	***
ml.lp	35	5.7×10^{-4}	3.9	***
residual	160	1.4×10^{-4}		

APPENDIX 3.5. Abbreviated ANOVA of results from Experiment 3.3.5a. Conductivity (siemens x 10^{-6}) of droplets on the leaf surfaces of three brassicas (cv) in relation to leaf position (lp).

	Hours after application of droplet to leaf surface											
	0				10				24			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
cv	2	6.3	1.8	ns	2	13.1	0.8	ns	2	17.1	0.6	ns
residual	18	3.6			18	16.2			18	30.2		
lp	5	8.2	2.8	*	5	16.7	2.0	ns	5	51.6	2.7	*
cv.lp	10	5.4	1.8	ns	10	7.4	0.9	ns	10	30.9	1.6	ns
residual	135	3.0			114 (21)	8.2			114 (21)	19.1		

APPENDIX 3.6. Abbreviated ANOVA of results from Experiment 3.3.5b. Conductivity (siemens x 10^{-6}) of droplets on the leaf surfaces of gemmifera mutants (ml) in relation to leaf position (lp).

	Hours after application of droplet to leaf surface											
	0				10				24			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
ml	7	24.9	3.3	**	7	248.7	7.0	***	7	388.4	6.2	***
residual	63	7.5			60 (3)	35.6			60 (3)	62.9		
lp	2	4.5	1.1	ns	2	112.6	3.9	*	2	551.9	14.7	***
ml.lp	14	5.7	1.3	ns	14	124.7	4.3	***	14	144.8	3.8	***
residual	144	4.3			112 (32)	29.0			112 (32)	37.6		

APPENDIX 4.1. Abbreviated ANOVA of results from Experiment 4.3.1. Lesion numbers of Alternaria brassicicola and Alternaria alternata and infection scores of Erysiphe cruciferarum on leaves of three brassicas (cv) in relation to leaf position (lp).

	Brassica pathogen											
	Alternaria brassicicola				*Alternaria alternata*				*Erysiphe cruciferarum*			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
cv	2	230445	2.0	ns	2	70338	5.0	*	2	33.6	111.2	***
residual	14	113396			14	13971			14	0.3		
lp	2	2190706	22.2	***	2	595470	53.2	***	2	4.1	10.1	***
cv.lp	4	678348	6.9	***	4	10283	0.9	ns	4	1.4	3.6	*
residual	41 (1)	98553			42	11180			42	19.1		

APPENDIX 4.2. Abbreviated ANOVA of results from Experiment 4.3.2. Lesion numbers of Alternaria brassicicola and infection scores of Alternaria alternata and Erysiphe cruciferarum on leaves of three brassicas (cv) in relation to leaf position (lp) and treatment with the commercial surfactant Agral.

	Brassica pathogen											
	Alternaria brassicicola				*Alternaria alternata*				*Erysiphe cruciferarum*			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
sf	1	4714432	13.6	*	1	90.2	35.9	**	1	25.2	43.6	**
residual	4	347968			4	2.5			4	0.6		
cv	2	240188	1.3	ns	2	7.0	2.0	ns	2	57.5	55.5	***
sf.cv	2	149915	0.8	ns	2	6.3	1.8	ns	2	22.7	21.9	***
residual	16	195622			16	3.5			16	1.0		
lp	5	1559692	10.6	***	5	62.5	20.4	***	5	4.4	6.3	***
sf.lp	5	666716	4.5	***	5	3.4	1.1	ns	5	1.7	2.4	*
cv.lp	10	433241	2.9	**	10	7.3	2.4	*	10	0.6	0.9	ns
sf.cv.lp	10	199147	1.4	ns	10	3.3	1.1	ns	10	2.8	4.0	***
residual	114 (6)	147658			116 (4)	3.1			119 (1)	0.7		

APPENDIX 4.3. Abbreviated ANOVA of results from Experiment 4.3.3. Infection scores of Alternaria brassicicola, Alternaria alternata and Erysiphe cruciferarum on leaves of three brassicas (cv) grown in different temperatures (temp) and light intensities (liin) and in relation to leaf position (lp).

	Brassica pathogen											
	Alternaria brassicicola				*Alternaria alternata*				*Erysiphe cruciferarum*			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
temp	1	55.0	11.7	*	1	2.7	2.4	ns	1	241.0	81.7	**
residual	4	4.7			4	1.1			4	3.0		
liin	1	20.3	16.9	**	1	0.1	0.0	ns	1	11.7	5.5	***
temp.liin	1	8.3	6.9	*	1	0.1	0.2	ns	1	6.2	2.9	***
residual	7 (1)	1.2			8	1.0			7 (1)	2.1		
cv	2	6.4	4.8	*	2	0.6	1.6	ns	2	22.3	25.5	***
temp.cv	2	26.2	19.5	***	2	0.5	1.4	ns	2	7.0	8.0	*
liin.cv	2	5.9	4.4	*	2	0.6	1.6	ns	2	0.1	0.1	ns
temp.liin.cv	2	12.0	8.9	***	2	0.3	0.9	ns	2	4.5	5.2	***
residual	30 (2)	1.3			30 (2)	0.4			30 (2)	0.9		

Appendix 4.3. continued.

	Brassica pathogen											
	Alternaria brassicicola				*Alternaria alternata*				*Erysiphe cruciferarum*			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
lp	5	69.0	52.0	***	5	1.0	3.3	**	5	20.8	35.7	***
temp.lp	5	2.6	2.0	ns	5	0.3	1.1	ns	5	8.3	14.3	***
liin.lp	5	3.3	2.5	*	5	0.3	0.9	ns	5	0.5	0.9	ns
cv.lp	10	11.0	8.2	***	10	0.7	2.3	*	10	2.1	3.6	***
temp.liin.lp	5	1.6	1.2	ns	5	0.3	1.0	ns	5	1.2	2.0	ns
temp.cv.lp	10	2.0	1.5	ns	10	0.2	0.6	ns	10	0.7	1.3	ns
liin.cv.lp	10	1.4	1.0	ns	10	0.3	1.0	ns	10	0.4	0.6	ns
temp.liin.cv.lp	10	5.3	4.0	***	10	0.2	0.8	ns	10	0.9	1.5	ns
residual	223 (17)	1.3			225 (15)	0.3			222 (18)	0.6		

APPENDIX 4.4. Abbreviated ANOVA of results from Experiment 4.3.4. Infection scores of Alternaria brassicicola, Alternaria alternata and Erysiphe cruciferarum on leaves of gemmifera mutants (ml) in relation to leaf position (lp).

	Brassica pathogen											
	Alternaria brassicicola				*Alternaria alternata*				*Erysiphe cruciferarum*			
Source of Variation	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig	df (mv)	ms	VR	sig
ml	7	22.7	9.6	***	7	3.15	2.3	ns	7	44.0	50.1	***
residual	28	2.4			28	1.35			28	0.9		
lp	5	36.1	18.7	***	5	11.6	9.5	***	5	8.3	14.5	***
ml.lp	35	4.8	2.5	***	35	2.3	1.9	**	35	2.4	4.2	***
residual	159 (1)	1.9			160	1.2			157 (3)	0.6		

Appendix 5.1. Preparation and staining of leaf disks for bright field and fluorescent microscopy.

For all staining procedures, specimens were first cleared in near boiling chloral hydrate (200% w/v). Then rinsed in three washes of sterile, distilled water.

Staining procedures.

Trypan blue: Specimens stained in 0.1% (w/v) trypan blue in glacial acetic acid/distilled water (44:55) for 2 - 5 minutes, rinsed in sterile, distilled water and mounted in 20% glycerol (Munro, 1985).

Aniline blue: Specimens stained in 0.1% (w/v) aniline blue in 0.1 M K_2PO_4 buffer (pH 9.2) for 2 - 4 hours in vacuum oven, then rinsed in sterile, distilled water and mounted in buffer solution (Munro, 1985).

Toluidine blue/trypan blue: Specimens immersed in 2 M sodium hydroxide for five minutes, rinsed in sterile, distilled water for two minutes then stained in 0.1% (w/v) toluidine blue in 0.1 M K_2PO_4 buffer (pH 6.5). After thirty minutes specimens rinsed in sterile, distilled water for two minutes, counter-stained in trypan blue as described above and mounted in 20% glycerol. (Ride & Pearce, 1979).

Aniline blue/trypan blue: Specimens stained in aniline blue as described above then rinsed in sterile, distilled water before counterstaining in trypan blue (see above) for 30 seconds and mounted in buffer solution.

APPENDIX 5.2. Abbreviated ANOVA of results from Experiment 5.3.1. Development of Alternaria brassicicola on leaves of three brassicas (cv) in relation to leaf position (lp).

Source of Variation	germination				germ-tube number				germ-tube length			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	13.0	0.7	ns	2	0.05	4.6	*	2	22.2	16.8	***
residual	8	18.5			8	0.01			8	1.3		
lp	2	54.6	2.6	ns	2	0.001	0.1	ns	2	44.1	69.4	***
cv.lp	4	38.5	1.8	ns	4	0.06	5.9	**	4	18.9	29.8	***
residual	21 (3)	21.1			21 (3)	0.01			21 (3)	0.6		
Source of Variation	branching				Axis divergence				Vesicle number			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	0.1	35.3	***	2	0.2	1.0	ns	2	0.1	3.3	ns
residual	8	0.003			8	0.2			8	0.004		
lp	2	0.1	34.3	***	2	0.2	2.0	ns	2	0.06	10.1	***
cv.lp	4	0.2	48.7	***	4	1.2	10.6	***	4	0.06	9.7	***
residual	21 (3)	0.004			21 (3)	0.1			21 (3)	0.006		

APPENDIX 5.2. continued.

Source of Variation	terminal vesicles df	ms	VR	sig	sub-cuticular hyphae df	ms	VR	sig
cv	2	242.4	5.0	*	2	368.2	1.0	ns
residual	8	48.2			8	354.0		
lp	2	518.6	7.4	**	2	2349.1	5.6	*
cv.lp	4	951.1	13.5	***	4	1170.6	2.8	*
residual	21 (3)	70.6			21 (3)	419.0		

Source of Variation	periclinal wall df	ms	VR	sig	anticlinal wall df	ms	VR	sig	guard cell area df	ms	VR	sig
cv	2	250.8	2.1	ns	2	260.7	1.7	ns	2	136.9	1.8	ns
residual	8	117.4			8	158.4			8	74.3		
lp	2	1144.1	9.1	**	2	850.8	5.07	*	2	62.9	1.3	ns
cv.lp	4	739.3	5.9	**	4	626.1	3.6	*	4	60.8	1.2	ns
residual	21 (3)	126.3			21 (3)	171.7			21 (3)	49.0		

APPENDIX 5.2. continued.

Source of Variation	normal SH				proximal distorted SH				distal distorted SH			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	157.8	0.5	ns	2	237.9	1.7	ns	2	59.9	0.8	ns
residual	8	296.4			8	136.8			8	78.1		
lp	2	2867.0	10.0	***	2	420.5	2.6	ns	2	1013.0	8.4	**
cv.lp	4	652.9	2.3	ns	4	553.9	3.5	*	4	72.9	0.6	ns
residual	21 (3)	285.8			21 (3)	159.9			21 (3)	120.9		

SH- sub-cuticular hyphae

APPENDIX 5.2. continued.

Source of Variation	SH - papilla df	ms	VR	sig	NSH - papilla df	ms	VR	sig	SH - no cell reaction df	ms	VR	sig
cv	2	154.5	2.8	ns	2	244.9	3.2	ns	2	880.3	28.0	***
residual	8	55.1			8	77.4			8	31.4		
lp	2	494.9	8.8	**	2	29.9	0.4	ns	2	549.9	6.0	**
cv.lp	4	16.9	0.3	ns	4	294.5	4.1	*	4	641.2	7.0	***
residual	21 (3)	56.6			21 (3)	72.0			21 (3)	92.3		

Source of Variation	NSH - no cell reaction df	ms	VR	sig	SH - necrosis df	ms	VR	sig	NSH - necrosis df	ms	VR	sig
cv	2	2215.8	13.3	**	2	2348.3	7.7	*	2	232.2	0.8	ns
residual	8	166.6			8	304.6			8	286.2		
lp	2	1087.3	10.0	***	2	165.2	0.7	ns	2	593.4	3.2	ns
cv.lp	4	401.9	4.0	*	4	1051.3	4.5	**	4	216.7	1.2	ns
residual	21 (3)	108.9			21 (3)	235.7			21 (3)	188.6		

SH- sub-cuticular hypha
NSH- no sub-cuticular hypha

APPENDIX 5.3. Abbreviated ANOVA of results from Experiment 5.3.1. Development of Alternaria alternata on leaves of three brassicas (cv) in relation to leaf position (lp).

Source of Variation	germination				germ-tube number				germ-tube length			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	30.7	2.1	ns	2	0.2	4.1	ns	2	16.9	12.8	**
residual	8	15.0			8	0.06			8	1.3		
lp	2	128.0	11.4	***	2	0.2	5.4	*	2	1.6	2.2	ns
cv.lp	4	63.8	5.7	**	4	0.2	7.0	***	4	7.7	10.8	***
residual	21 (3)	11.2			21 (3)	0.03			21 (3)	0.7		
Source of Variation	branching				Axis divergence				Vesicle number			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	0.03	0.4	ns	2	0.3	1.7	ns	2	0.5	13.5	**
residual	8	0.06			8	0.2			8	0.04		
lp	2	0.1	2.6	ns	2	0.9	6.3	**	2	0.02	0.5	ns
cv.lp	4	0.2	3.0	*	4	0.6	4.6	**	4	0.06	1.9	ns
residual	21 (3)	0.06			21 (3)	0.1			21 (3)	0.03		

APPENDIX 5.3. continued.

Source of Variation	terminal vesicles df	ms	VR	sig	sub-cuticular hyphae df	ms	VR	sig
cv	2	4294.7	16.8	**	2	127.7	2.9	ns
residual	8	255.7			8	44.0		
lp	2	1246.6	10.9	***	2	144.0	4.0	*
cv.lp	4	208.7	1.8	ns	4	131.1	3.7	*
residual	21 (3)	114.6			21 (3)	35.7		

Source of Variation	periclinal wall df	ms	VR	sig	anticlinal wall df	ms	VR	sig	guard cell area df	ms	VR	sig
cv	2	25.6	0.2	ns	2	47.3	0.3	ns	2	115.2	4.1	ns
residual	8	145.9			8	150.2			8	28.4		
lp	2	1693.6	10.1	***	2	2219.6	10.7	***	2	132.0	3.1	ns
cv.lp	4	98.0	0.6	ns	4	78.9	0.4	ns	4	44.4	1.0	ns
residual	21 (3)	167.2			21 (3)	207.4			21 (3)	42.3		

APPENDIX 5.3. continued.

Source of Variation	normal SH				proximal distorted SH				distal distorted SH			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	5287.0	2.1	ns	2	0.0	0.0		2	31.2	1.0	ns
residual	8	2509.0			8	0.0			8	31.2		
lp	2	9534.0	6.8	**	2	0.0	0.0		2	10.4	0.9	ns
cv.lp	4	2271.0	1.6	ns	4	0.0	0.0		4	10.4	0.9	ns
residual	21 (3)	1397.0			21 (3)	0.0			21 (3)	11.9		

SH- sub-cuticular hyphae

APPENDIX 5.3. continued.

Source of Variation	SH + papilla df	ms	VR	sig	NSH + papilla df	ms	VR	sig	SH - cell reaction df	ms	VR	sig
cv	2	0.0	0.0		2	1680.1	11.8	**	2	0.0	0.0	
residual	8	0.0			8	142.4			8	0.0		
lp	2	0.0	0.0		2	312.7	3.8	*	2	0.0	0.0	
cv.lp	4	0.0	0.0		4	175.2	2.0	ns	4	0.0	0.0	
residual	21 (3)	0.0			21 (3)	85.3			21 (3)	0.0		

Source of Variation	NSH - cell reaction df	ms	VR	sig	SH + necrosis df	ms	VR	sig	NSH + necrosis df	ms	VR	sig
cv	2	5332.6	23.4	***	2	124.5	5.8	*	2	536.3	6.8	*
residual	8	227.6			8	21.4			8	78.9		
lp	2	4829.8	17.8	***	2	160.4	4.4	*	2	2080.1	17.9	***
cv.lp	4	746.6	2.8	ns	4	139.7	3.8	*	4	40.0	0.3	ns
residual	21 (3)	271.4			21 (3)	36.8			21 (3)	116.5		

SH- sub-cuticular hypha
NSH- no sub-cuticular hypha

APPENDIX 5.4. Abbreviated ANOVA of results from Experiment 5.3.2. Development of Alternaria brassicicola on leaves of gemmifera mutants (ml).

Source of Variation	df (mv)		ms	VR	sig
(a) germination					
ml	7		65.0	3.2	*
residual	27	(1)	20.4		
(b) germ-tube number					
ml	7		0.1	3.2	*
residual	27	(1)	0.04		
(c) germ-tube length					
ml	7		1.0	2.1	ns
residual	27	(1)	0.5		
(d) branching					
ml	7		0.02	4.3	**
residual	27	(1)	0.005		
(e) axis divergence					
ml	7		0.7	5.6	***
residual	27	(1)	0.1		
(f) vesicle number					
ml	7		0.02	3.2	*
residual	27	(1)	0.007		

APPENDIX 5.4. continued.

Source of Variation	df (mv)		ms	VR	sig
(a) terminal vesicles					
ml	7		109.7	8.3	***
residual	27	(1)	13.2		
(b) periclinal wall					
ml	7		62.2	2.1	ns
residual	27	(1)	30.0		
(c) anticlinal wall					
ml	7		126.7	2.3	ns
residual	27	(1)	54.2		
(d) guard cell area					
ml	7		57.9	1.9	ns
residual	27	(1)	30.0		

APPENDIX 5.4. continued.

Source of Variation	df (mv)		ms	VR	sig
(a) sub-cuticular hyphae					
ml	7		732.1	8.2	***
residual	27	(1)	89.7		
(b) normal sub-cuticular hyphae					
ml	7		1605.1	7.5	***
residual	27	(1)	214.6		
(c) proximal distorted sub-cuticular hyphae					
ml	7		708.0	4.8	**
residual	27	(1)	149.0		
(d) distal distorted sub-cuticular hyphae					
ml	7		262.4	1.7	ns
residual	27	(1)	150.7		

APPENDIX 5.4. continued.

Source of Variation	df (mv)		ms	VR	sig
(a) SH + papillae					
ml	7		117.6	1.4	ns
residual	27	(1)	85.1		
(b) NSH + papillae					
ml	7		112.3	3.5	**
residual	27	(1)	32.1		
(c) SH - cell reaction					
ml	7		309.7	10.0	***
residual	27	(1)	30.9		
(d) NSH - cell reaction					
ml	7		924.6	7.7	***
residual	27	(1)	120.5		
(e) SH + necrosis					
ml	7		1710.6	10.0	***
residual	27	(1)	171.2		
(f) NSH + necrosis					
ml	7		138.6	1.7	ns
residual	27	(1)	81.9		

SH- sub-cuticular hyphae

NSH- no sub-cuticular hyphae

APPENDIX 5.5. Abbreviated ANOVA of results from Experiment 5.3.2. Development of Alternaria alternata on leaves of gemmifera mutants (ml).

Source of Variation	df (mv)		ms	VR	sig
(a) germination					
ml	7		7.6	1.1	ns
residual	26	(2)	7.1		
(b) germ-tube number					
ml	7		0.1	5.8	***
residual	26	(2)	0.02		
(c) germ-tube length					
ml	7		9.3	6.0	***
residual	26	(2)	1.6		
(d) branching					
ml	7		0.06	2.1	ns
residual	26	(2)	0.03		
(e) axis divergence					
ml	7		0.6	4.1	**
residual	26	(2)	0.2		
(f) vesicle number					
ml	7		0.5	6.6	***
residual	26	(2)	0.07		

APPENDIX 5.5. continued.

Source of Variation	df (mv)		ms	VR	sig
(a) terminal vesicles					
ml	7		714.2	3.2	*
residual	26	(2)	225.8		
(b) periclinal wall					
ml	7		214.8	1.8	ns
residual	26	(2)	117.7		
(c) anticlinal wall					
ml	7		132.8	0.9	ns
residual	26	(2)	150.1		
(d) guard cell area					
ml	7		70.9	1.7	ns
residual	26	(2)	42.1		

APPENDIX 5.5. contiued.

Source of Variation	df (mv)		ms	VR	sig
(a) sub-cuticular hyphae					
ml	7		719.8	3.2	*
residual	26	(2)	227.2		
(b) normal sub-cuticular hyphae					
ml	7		3265.0	1.5	ns
residual	26	(2)	2233.0		
(d) proximal distorted sub-cuticular hyphae					
ml	7		263.1	0.7	ns
residual	26	(2)	363.2		
(e) distal distorted sub-cuticular hyphae					
ml	7		9.9	0.8	ns
residual	27	(1)	12.0		

APPENDIX 5.5. contiued.

Source of Variation	df (mv)		ms	VR	sig
(a) SH + papillae					
ml	7		34.5	6.9	***
residual	27	(1)	5.0		
(b) NSH + papillae					
ml	7		62.3	0.7	ns
residual	27	(1)	84.2		
(c) SH - cell reaction					
ml	7		0.0	0.0	
residual	27	(1)	0.0		
(d) NSH - cell reaction					
ml	7		746.3	2.0	ns
residual	27	(1)	379.7		
(e) SH + necrosis					
ml	7		270.4	1.4	ns
residual	27	(1)	194.8		
(f) NSH + necrosis					
ml	7		228.7	3.2	*
residual	27	(1)	71.6		

SH- sub-cuticular hyphae

NSH- no sub-cuticular hyphae

APPENDIX 5.6. Abbreviated ANOVA of results from Experiment 5.3.3. Development of Erysiphe cruciferarum on leaves of three brassicas (cv) in relation to leaf position (lp).

Source of Variation	germination df		ms	VR	sig	germ-tube length df		ms	VR	sig
cv	2		32.3	0.6	ns	2		0.02	4.8	*
residual	8		58.8			8		0.004		
lp	2		61.6	0.6	ns	2		0.05	4.5	*
cv.lp	4		60.8	0.6	ns	4		0.009	0.8	ns
residual	22	(2)	102.7			22	(2)	0.01		
Source of Variation	appressoria df		ms	VR	sig	appressorial size df		ms	VR	sig
cv	2		15.4	0.7	ns	2		0.5	1.8	ns
residual	8		23.6			8		0.3		
lp	2		6.6	0.3	ns	2		0.3	0.9	ns
cv.lp	4		13.8	0.6	ns	4		0.7	2.5	*
residual	22	(2)	22.4			22	(2)	0.3		

APPENDIX 5.6. continued.

Source of Variation	appressorial aborts df	ms	VR	sig	secondary structures df	ms	VR	sig
cv	2	1038.0	6.0	*	2	703.4	5.5	*
residual	8	174.4			8	128.3		
lp	2	522.9	4.2	*	2	736.8	4.3	*
cv.lp	4	114.0	0.9	ns	4	168.8	1.0	ns
residual	22 (2)	124.8			22 (2)	170.5		

Source of Variation	periclinal wall df	ms	VR	sig	anticlinal wall df	ms	VR	sig	guard cell area df	ms	VR	sig
cv	2	893.7	10.5	**	2	460.3	11.3	**	2	32.0	2.6	ns
residual	8	84.8			8	40.6			8	12.2		
lp	2	144.1	1.3	ns	2	150.8	1.6	ns	2	1.5	0.1	ns
cv.lp	4	427.3	4.1	*	4	356.7	3.8	*	4	0.9	0.1	ns
residual	22 (2)	113.0			22 (2)	93.6			22 (2)	12.0		

APPENDIX 5.6. continued.

Source of Variation	secondary stuctures + papillae df	ms	VR	sig	secondary structures - papillae df	ms	VR	sig
cv	2	1407.3	4.8	*	2	0.4	0.01	ns
residual	8	290.9			8	23.0		
lp	2	551.5	2.8	ns	2	34.3	1.0	ns
cv.lp	4	168.4	0.9	ns	4	10.3	0.3	ns
residual	22 (2)	195.5			22 (2)	33.3		
Source of Variation	no secondary structures + papillae df	ms	VR	sig	no secondary structures - papillae df	ms	VR	sig
cv	2	240.9	1.0	ns	2	465.1	3.7	ns
residual	8	232.0			8	125.9		
lp	2	212.6	3.3	ns	2	292.2	3.3	ns
cv.lp	4	160.8	2.5	ns	4	310.1	3.5	*
residual	22 (2)	64.4			22 (2)	87.5		

APPENDIX 5.6. continued: average numbers of germ-tubes, branches and conidial intials on conidia.

Source of Variation	average germ-tubes				average branches				average conidial initials			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	2.6	4.8	*	2	3.7	13.0	**	2	0.8	18.1	***
residual	8	0.5			8	0.3			8	0.04		
lp	2	3.5	12.2	***	2	4.7	11.1	***	2	0.5	5.6	*
cv.lp	4	1.1	4.0	*	4	0.4	1.0	ns	4	0.5	4.9	**
residual	22 (2)	0.3			22 (2)	0.4			22 (2)	11.9		

APPENDIX 5.6. continued: numbers of germ-tubes on conidia.

Source of Variation	1 df	ms	VR	sig	2 df	ms	VR	sig	3 df	ms	VR	sig
cv	2	74.2	1.0	ns	2	88.3	0.7	ns	2	171.2	1.0	ns
residual	8	71.3			8	121.4			8	169.5		
lp	2	48.4	1.0	ns	2	475.2	6.2	**	2	793.8	8.2	**
cv.lp	4	67.5	1.4	ns	4	100.2	1.3	ns	4	157.7	1.6	ns
residual	22 (2)	46.7			22 (2)	77.0			22 (2)	96.9		
Source of Variation	4 df	ms	VR	sig	5 df	ms	VR	sig	6 df	ms	VR	sig
cv	2	803.7	9.8	ns	2	32.2	0.2	ns	2	2840.0	6.6	*
residual	8	81.7			8	216.9			8	428.6		
lp	2	156.8	0.7	ns	2	309.3	2.2	ns	2	2590.9	12.3	**
cv.lp	4	564.6	2.4	ns	4	185.9	1.3	ns	4	1593.9	7.5	ns
residual	22 (2)	233.4			22 (2)	144.0			22 (2)	211.4		

APPENDIX 5.6. continued: positions of germ-tubes on conidia.

Source of Variation	C				A				D			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	108.2	0.8	ns	2	161.4	2.9	ns	2	312.4	1.2	ns
residual	8	131.7			8	56.4			8	169.5		
lp	2	283.5	3.4	ns	2	171.0	4.7	*	2	1747.4	9.3	**
cv.lp	4	82.6	2.8	ns	4	45.7	1.3	ns	4	271.7	1.4	ns
residual	22 (2)	46.7			22 (2)	36.4			22 (2)	187.7		
Source of Variation	B				X				Y			
	df	ms	VR	sig	df	ms	VR	sig	df	ms	VR	sig
cv	2	1395.1	2.5	ns	2	1887.0	4.5	*	2	607.8	1.3	NS
residual	8	556.5			8	419.7			8	470.9		
lp	2	657.5	3.4	ns	2	1113.0	2.3	ns	2	5938.5	24.9	***
cv.lp	4	221.6	1.1	ns	4	3127.1	6.5	***	4	1368.2	5.7	***
residual	22 (2)	195.1			22 (2)	144.0			22 (2)	238.5		

APPENDIX 5.6. continued: numbers of branches on conidia.

Source of Variation	0 df	ms	VR	sig	1 df	ms	VR	sig
cv	2	1741.3	4.0	ns	2	299.9	2.5	ns
residual	8	442.5			8	117.9		
lp	2	2112.0	8.0	**	2	761.0	4.7	*
cv.lp	4	78.2	0.3	ns	4	79.5	0.5	ns
residual	22 (2)	263.5			22 (2)	193.4		

Source of Variation	2 df	ms	VR	sig	3 df	ms	VR	sig	4 df	ms	VR	sig
cv	2	319.6	5.3	*	2	256.8	1.6	ns	2	5649.2	19.6	***
residual	8	60.0			8	165.5			8	288.5		
lp	2	151.1	1.2	ns	2	205.5	1.7	ns	2	4650.1	9.2	**
cv.lp	4	101.0	0.8	ns	4	129.6	1.1	ns	4	628.7	1.2	ns
residual	22 (2)	127.8			22 (2)	123.1			22 (2)	505.0		

APPENDIX 5.6. continued: positions of branches on conidia.

Source of Variation	0 df	ms	VR	sig	C df	ms	VR	sig
cv	2	1741.3	4.0	ns	2	3071.0	4.1	ns
residual	8	442.5			8	756.9		
lp	2	2112.0	8.0	**	2	4595.1	6.2	**
cv.lp	4	78.2	0.3	ns	4	282.1	0.4	ns
residual	22 (2)	263.5			22 (2)	742.6		

Source of Variation	A df	ms	VR	sig	D df	ms	VR	sig	B df	ms	VR	sig
cv	2	2196.2	5.9	*	2	4761.5	19.4	***	2	4959.0	6.7	*
residual	8	373.2			8	246.0			8	738.1		
lp	2	1594.6	4.9	*	2	6324.5	14.0	***	2	3548.7	7.8	**
cv.lp	4	212.2	0.7	ns	4	196.2	0.4	ns	4	738.7	1.7	ns
residual	22 (2)	323.0			22 (2)	450.7			22 (2)	456.2		

APPENDIX 5.6. continued: numbers on conidial initials on conidia.

Source of Variation	0 df	ms	VR	sig	1 df	ms	VR	sig
cv	2	2462.5	14.7	**	2	872.6	4.9	*
residual	8	167.8			8	176.9		
lp	2	2368.2	10.9	***	2	187.0	0.8	ns
cv.lp	4	1421.2	6.5	**	4	433.3	2.0	ns
residual	22 (2)	217.2			22 (2)	220.9		

Source of Variation	2 df	ms	VR	sig	3 df	ms	VR	sig	4 df	ms	VR	sig
cv	2	241.4	7.2	*	2	57.2	2.2	ns	2	445.0	11.2	**
residual	8	33.4			8	26.9			8	39.9		
lp	2	29.8	0.5	ns	2	91.0	2.7	ns	2	378.2	6.1	**
cv.lp	4	139.5	2.4	ns	4	35.1	1.1	ns	4	378.2	6.1	**
residual	22 (2)	67.3			22 (2)	33.2			22 (2)	62.3		

APPENDIX 5.6. continued: positions of conidia initials on conidia.

Source of Variation	0 df	ms	VR	sig	C^{GT} df	ms	VR	sig
cv	2	2486.4	13.0	**	2	666.6	5.4	*
residual	8	191.2			8	124.4		
lp	2	2242.0	9.6	***	2	1417.3	15.5	***
cv.lp	4	1543.0	6.6	**	4	862.1	9.4	***
residual	22 (2)	233.1			22 (2)	91.3		

Source of Variation	C^{BR} df	ms	VR	sig	A^{GT} df	ms	VR	sig	A^{BR} df	ms	VR	sig
cv	2	801.1	5.4	*	2	1217.7	17.5	**	2	1278.4	10.1	**
residual	8	148.4			8	69.7			8	126.1		
lp	2	776.1	6.3	**	2	1000.9	7.9	**	2	1200.6	10.0	***
cv.lp	4	410.0	3.3	*	4	1161.3	9.1	***	4	565.5	4.7	**
residual	22 (2)	123.6			22 (2)	127.2			22 (2)	119.8		

GT - conidial initial on germ-tube; BR - conidial intial on branch

APPENDIX 5.6. continued: positions of conidial initials on conidia.

Source of Variation	D^{GT} df	ms	VR	sig	D^{BR} df	ms	VR	sig
cv	2	1119.0	12.4	**	2	712.5	18.2	**
residual	8	90.1			8	39.2		
lp	2	356.3	2.6	ns	2	660.9	5.0	**
cv.lp	4	354.7	2.6	ns	4	474.1	3.6	**
residual	22 (2)	136.0			22 (2)	133.3		
Source of Variation	B^{GT} df	ms	VR	sig	B^{BR} df	ms	VR	sig
cv	2	1137.9	28.1	***	2	473.4	7.7	*
residual	8	40.4			8	61.4		
lp	2	501.7	4.0	*	2	403.6	4.2	*
cv.lp	4	556.0	4.5	*	4	402.9	4.2	*
residual	22 (2)	124.0			22 (2)	95.1		

GT - conidial initial on germ-tube; BR - conidial intial on branch

APPENDIX 5.7. Abbreviated ANOVA of results from Experiment 5.3.4. Development of Erysiphe cruciferarum on leaves of gemmifera mutants (ml).

Source of Variation	df (mv)	ms	VR	sig
(a) germination				
ml	7	487.7	4.3	**
residual	28	112.7		
(b) germ-tube length				
ml	7	0.06	2.0	ns
residual	28	0.03		
(c) appressoria				
ml	7	299.4	4.7	**
residual	28	63.8		
(d) appressorial size				
ml	7	2.8	13.0	***
residual	28	0.2		

APPENDIX 5.7. continued.

Source of Variation	df (mv)	ms	VR	sig
(a) periclinal wall				
ml	7	528.4	10.3	***
residual	28	51.9		
(b) anticlinal wall				
ml	7	398.8	5.9	***
residual	28	68.0		
(c) guard cell area				
ml	7	20.2	2.1	ns
residual	28	9.6		
(d) abortive appressoria				
ml	7	1332.3	10.5	***
residual	28	126.4		
(e) secondary structures				
ml	7	1890.1	11.2	***
residual	28	169.2		

APPENDIX 5.7. continued.

Source of Variation	df (mv)	ms	VR	sig
(a) secondary structures + papillae				
ml	7	3004.8	11.9	***
residual	28	252.9		
(b) secondary structures - papillae				
ml	7	338.5	2.5	*
residual	28	137.0		
(c) no secondary structures + papillae				
ml	7	400.5	2.3	ns
residual	28	172.0		
(d) no secondary structures - papillae				
ml	7	777.3	5.5	***
residual	28	141.3		

APPENDIX 5.7. continued: average numbers of germ-tubes and branches on conidia of Erysiphe cruciferarum.

Source of Variation	df (mv)	ms	VR	sig
(a) germ-tubes				
ml	7	2.1	5.5	***
residual	28	0.4		
(b) branches				
ml	7	0.5	8.8	***
residual	28	0.06		

APPENDIX 5.7. continued: numbers of germ-tubes on conidia.

Source of Variation	df (mv)	ms	VR	sig
(a) 1				
ml	7	34.0	0.9	ns
residual	28	41.1		
(b) 2				
ml	7	247.9	1.4	ns
residual	28	177.9		
(c) 3				
ml	7	703.4	3.3	*
residual	28	213.8		
(d) 4				
ml	7	193.9	0.6	ns
residual	28	321.8		
(e) 5				
ml	7	701.7	3.9	**
residual	28	182.0		
(f) 6				
ml	7	244.5	2.4	*
residual	28	101.5		

APPENDIX 5.7. continued: position of germ-tubes on conidia.

Source of Variation	df (mv)	ms	VR	sig
(a) C				
ml	7	245.0	0.9	ns
residual	28	259.2		
(b) A				
ml	7	935.5	3.5	**
residual	28	266.1		
(c) D				
ml	7	821.8	2.4	*
residual	28	346.8		
(d) B				
ml	7	1361.4	5.9	***
residual	28	229.3		
(e) X				
ml	7	724.0	2.8	*
residual	28	262.2		
(f) Y				
ml	7	1012.5	3.6	**
residual	28	281.6		

APPENDIX 5.7. continued: numbers of branches on germ-tubes on conidia.

Source of Variation	df (mv)	ms	VR	sig
(a) 0				
ml	7	1187.1	2.6	*
residual	28	451.5		
(b) 1				
ml	7	171.7	0.5	ns
residual	28	371.6		
(c) 2				
ml	7	482.9	3.5	**
residual	28	137.9		
(d) 3				
ml	7	56.7	2.8	*
residual	28	20.0		
(e) 4				
ml	7	94.0	3.7	**
residual	28	25.2		

APPENDIX 5.7. continued: positions of branches on germ-tubes on conidia.

Source of Variation	df (mv)	ms	VR	sig
(a) 0				
ml	7	1187.1	2.6	*
residual	28	451.5		
(b) C				
ml	7	645.9	5.2	***
residual	28	123.4		
(c) A				
ml	7	11729.8	3.1	*
residual	28	377.1		
(d) D				
ml	7	542.6	3.8	**
residual	28	142.7		
(e) B				
ml	7	660.4	6.7	***
residual	28	98.4		

APPENDIX 5.8. Abbreviated ANOVA of results from Experiment 5.3.5. Development of Erysiphe cruciferarum on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) germination					
ho	1		55.4	0.4	ns
residual	6	(1)	125.5		
(b) germ-tube length					
ho	1		0.1	4.6	ns
residual	6	(1)	0.02		
(c) abortive germ-tubes					
ho	1		3.6	1.1	ns
residual	6	(1)	3.2		
(d) normal appressoria					
ho	1		23511.9	164.9	***
residual	6	(1)	142.6		

APPENDIX 5.8. continued: development of Erysiphe cruciferarum on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) periclinal wall					
ho	1		2178.1	10.4	*
residual	6	(1)	209.3		
(b) anticlinal wall					
ho	1		1524.4	4.5	ns
residual	6	(1)	341.5		
(c) guard cell area					
ho	1		32.6	1.5	ns
residual	6	(1)	21.1		
(d) secondary structures					
ho	1		21910.4	537.4	***
residual	6	(1)	40.8		

APPENDIX 5.8. continued: development of Erysiphe cruciferarum on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) normal haustoria + papilla					
ho	1		15324.4	72.1	***
residual	6	(1)	212.7		
(b) encapsulated haustoria					
ho	6	(1)	1175.1	6.7	*
residual	6	(1)	176.7		
(c) haustoria without wall apposition					
ho	1		58.0	4.2	ns
residual	6	(1)	13.7		
(d) papillae without haustoria					
ho	1		928.8	4.5	ns
residual	6	(1)	205.2		
(e) no papillae and no haustoria					
ho	1		4701.9	51.1	***
residual	6	(2)	92.1		

APPENDIX 5.8. continued: development of Erysiphe cruciferarum on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) no cell reaction and no secondary structures					
ho	1		17777.6	82.8	***
residual	6	(1)	214.8		
(b) no cell reaction with secondary structures					
ho	1		293.6	8.4	*
residual	6	(1)	34.9		
(c) degenerate cells with no secondary structures					
ho	1		130.8	1.1	ns
residual	6	(1)	121.7		
(d) degenerate cells with secondary structures					
ho	1		15803.1	126.6	***
residual	6	(1)	124.8		

APPENDIX 5.8. continued. development of Erysiphe graminis on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) germination					
ho	1		179.2	9.6	*
residual	6	(1)	18.8		
(b) germ-tube length					
ho	1		0.008	7.4	*
residual	6	(1)	0.001		
(c) primary germ-tubes					
ho	1		0.5	4.2	ns
residual	6	(1)	0.1		
(d) abortive germ-tubes					
ho	1		1044.0	36.5	***
residual	6	(1)	28.6		
(e) normal appressoria					
ho	1		1911.9	17.5	**
residual	6	(1)	109.0		

APPENDIX 5.8. continued: development of Erysiphe graminis on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) periclinal wall					
ho	1		443.0	0.9	ns
residual	6	(1)	506.2		
(b) anticlinal wall					
ho	1		404.3	0.8	ns
residual	6	(1)	485.0		
(c) guard cell area					
ho	1		3.6	1.1	ns
residual	6	(1)	3.2		
(d) secondary structures					
ho	1		1619.3	21.8	**
residual	6	(1)	74.2		

APPENDIX 5.8. continued: development of Erysiphe graminis on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) normal haustoria + papilla					
ho	1		1966.0	47.4	***
residual	6	(1)	41.5		
(b) encapsulated haustoria					
ho	6		598.8	2.9	ns
residual	6	(1)	208.1		
(c) haustoria without wall apposition					
ho	1		0.0	0.0	
residual	6	(1)	0.0		
(d) papillae without haustoria					
ho	1		4.8	0.01	ns
residual	6	(1)	466.0		
(e) no papillae and no haustoria					
ho	1		2830.8	39.8	***
residual	6	(1)	71.2		

APPENDIX 5.8. continued: development of Erysiphe graminis on leaves of different hosts (ho).

Source of Variation	df (mv)		ms	VR	sig
(a) no cell reaction and no secondary structures					
ho	1		3852.0	5.9	ns
residual	6	(1)	654.9		
(b) no cell reaction with secondary structures					
ho	1		19.6	3.3	ns
residual	6	(1)	34.9		
(c) degenerate cells with no secondary structures					
ho	1		20.0	0.02	ns
residual	6	(1)	1117.0		
(d) degenerate cells with secondary structures					
ho	1		4422.81	45.2	**
residual	6	(1)	97.8		